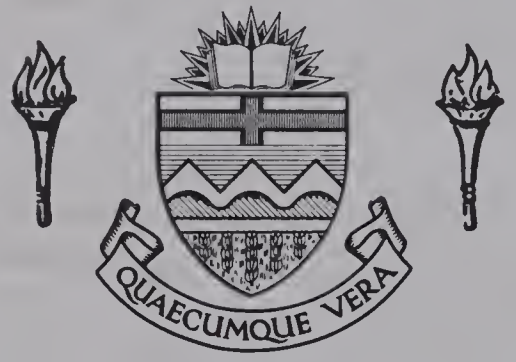

THE UNIVERSITY OF ALBERTA

ARGININE PHOSPHOKINASE FROM HONEYBEES

by

© ALOYSIUS CHI-FAI CHEUNG

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled ARGININE PHOSPHOKINASE FROM HONEYBEES submitted by Aloysius Chi-Fai Cheung in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

Arginine phosphokinase was purified from honeybee thoraces by ammonium sulfate fractionation, Sephadex G-100 gel chromatography, and DEAE-cellulose ion-exchange chromatography. Its molecular weight was estimated by Sephadex gel chromatography to be 36,000 ± 3,000. Enzymic activity was unaffected by incubation between 30 and 40 C for 15 min. Addition of 2-mercapto-ethanol, reduced glutathion, or cysteine activated the enzyme by as much as 33%. The enzyme was activated to various extents by Mg^{++}, Mn^{++}, Ca^{++}, Co^{++}, and Cu^{++}. The nucleotides UTP, GTP, CTP, and ADP could not substitute for ATP as substrate. Besides L-arginine, the enzyme phosphorylated L-arginine methyl ester and to a much lesser extent D-arginine, but did not phosphorylate creatine, guanidoacetic acid, nor hippuryl-L-arginine.

In the direction of AP synthesis, the enzyme had a pH optimum around 8.3. The energy of activation for the reaction over the range 22-39 C was about 7,500 cal/mole. The optimum ratio of Mg^{++}:ATP was about 1:1.

In the direction of ATP synthesis, the pH optimum was around pH 7.2 and the activation energy over the range 18-44 C was about 10,500 cal/mole. The optimum ratio of Mg^{++}:ADP was about 4:1.

The initial velocities of the reactions in the direction of ATP synthesis and AP synthesis were measured at varying concentrations of one substrate while the concentration of the other substrate was held constant at several levels. The double reciprocal plots of the data thus obtained yielded a series of intersecting lines, indicating that the enzyme has a sequential mechanism. Product inhibition studies showed that arginine was competitive with AP and non-competitive with ADP; whereas ATP was competitive with ADP and non-competitive with arginine. The results from initial velocity and product inhibition studies suggested that the enzyme has a

rapid equilibrium, random mechanism.

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LIST OF ABBREVIATIONS

ADP	-	adenosine diphosphate
AP	-	arginine phosphate
APK	-	arginine phosphokinase
Arg	-	arginine
ATP	-	adenosine triphosphate
BaAP	-	barium arginine phosphate
CP	-	creatine phosphate
CTP	-	cytosine triphosphate
DEAE-cellulose	-	diethyl aminoethyl cellulose
EDTA	-	disodium ethylenediamine-tetraacetate
G-6-P DH	-	glucose-6-phosphate dehydrogenase
GTP	-	guanosine triphosphate
NADP	-	nicotinamide-adenine dinucleotide phosphate
SAS	-	saturated ammonium sulfate
-SH	-	sulfhydryl
Tris	-	tris(hydroxymethyl)aminomethane
UTP	-	uridine triphosphate

1. INTRODUCTION

When a muscle becomes active, its demand for energy increases rapidly. In order to meet the demand, ATP in the muscle is supplemented by a store of secondary phosphagen, the phosphate group of which can readily be transferred to ADP to form ATP. However, the energy available from the stored ATP and the secondary phosphagen in the muscle is limited. For sustained activity, energy has to be derived from carbohydrate, fat, protein, or a combination of these. In vertebrates the phosphagen is creatine phosphate whilst in invertebrates it is mainly arginine phosphate. In insect muscle arginine phosphate is the only secondary phosphagen found. Arginine phosphokinase catalyzes the following reaction:

$$ADP + AP + H^{+} \rightleftharpoons ATP + \text{arginine}.$$

APK was first discovered by Lohmann (1935) using extracts from crab muscle. Since then, APK has been purified or identified from extracts of bacteria (Di Jeso, 1967), protozoans (Robin and Viala, 1966; Watts and Bannister, 1970), molluscs (Virden and Watts, 1964), annelids (Regnouf *et al.*, 1969; Robin *et al.*, 1969), horseshoe crabs, arachnids (Blethan and Kaplan, 1968), crustaceans (Elodi and Szorényi, 1956; Morrison *et al.*, 1957; Virden and Watts, 1964; Pradel *et al.*, 1964; Virden *et al.*, 1965; and Blethan and Kaplan, 1967, 1968), echinoderms (Griffiths *et al.*, 1957a; Virden and Watts, 1964), tunicates, cephalochordates (Virden and Watt, 1964), and insects *Melanoplus bruneri*, *Apis mellifera*, *Porthetria dispar*, *Sympetrum rubicundulum* (Blethan and Kaplan, 1968), and *Calliphora erythrocephala* (Lewis and Fowler, 1962).

APK in lobsters, crabs, insects, and many molluscs is a monomer with a molecular weight of 40,000 (Elodi and Szorényi, 1956; Virden *et al.*, 1966; Moreland and Watts, 1967; Blethan and Kaplan, 1968; Regnouf *et al.*,

1969; Oriol *et al.*, 1970; Cheung, 1971); APK in sea urchins, sipunculids, echinoderms, and some molluscs is a dimer with a molecular weight of 80,000 (Thoai *et al.*, 1966; Moreland *et al.*, 1967; Moreland and Watts, 1967); and APK in polychaetes is a tetramer with a molecular weight of 160,000 (Robin *et al.*, 1969).

Der Terrossian *et al.* (1966) determined the amino acid composition of APK from the lobster *Homarus vulgaris*. At the active center, there is probably an essential cysteine, lysine, and histidine residue (Pradel *et al.*, 1964; Virden and Watts, 1966; Kassab *et al.*, 1967, 1968; and Pradel and Kassab, 1968). There is also a tyrosine residue which is essential for maintaining the conformation of the active center (Kassab *et al.*, 1970). Der Terrossian *et al.* (1969) have shown that the structures of the active centers of lobster APK and rabbit creatine kinase are similar.

The properties of APK have been studied using enzymes from crabs, horseshoe crabs (Blethan and Kaplan, 1968), sea crayfish (Morrison *et al.*, 1957), lobsters (Virden *et al.*, 1965), and honeybees (Cheung, 1971). The kinetic mechanism of crustacean APK is a rapid equilibrium, random mechanism (Griffiths *et al.*, 1957b; Smith and Morrison, 1969; and Virden *et al.*, 1965). The distribution of guanidine kinases in echinoderms has been studied by Griffiths *et al.* (1957a), and in coelenterates, platyhelminths, annelids, echinoderms, tunicates and cephalochordates by Virden and Watts (1964).

2. STATEMENT OF THE PROBLEM

Little work has been done on arginine phosphokinase from insect sources. Recently Carlson *et al.* (1971) reported the crystallization and Cheung (1971) reported some of the properties of APK from honeybee thoraces. No detailed studies of an insect APK have been reported. The significance of APK in insect muscle activity, particularly in flight muscle activity, has not been investigated. Considering the rate at which ATP is required when the muscle is activated, the lengthy processes involved in glycolysis and the Krebs cycle before ATP is generated, I believe the energy stored in AP plays an important role in maintaining the ATP level in the initial phase of muscular activity. The purpose of the present work is to study the kinetic mechanism of APK purified from bee thoraces and factors that may affect the enzyme activity. Using the information obtained, I hope to suggest how the enzyme performs its function *in vivo*.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Experimental animals

Honeybees (*Apis mellifera* L.) were obtained from a local apiary. The bees were killed in the fall of the year by placing them in a container of dry ice and they were kept frozen until use.

3.1.2. Chemicals

ATP and L-arginine came from both Sigma Chemical and Calbiochem. G-6-P DH, UTP, CTP, GTP, ADP, L-arginine methyl ester, hippuryl-L-arginine, guanidoacetic acid, creatine, cytochrome c, Tris, and DEAE-cellulose came from Sigma Chemical. D-arginine, myoglobin, haemoglobin, and bovine albumin came from Nutritional Biochemicals. L-cysteine HCl, 2-mercaptoethanol, reduced glutathion, γ-globulin, and NADP came from Calbiochem. $MgSO_4$, $CuSO_4$, $MnSO_4$, $CoCl_2$, $CaCl_2$, ammonium molybdate, 1-amino-2-naphthol-4-sulfonic acid, and EDTA came from Fisher Scientific. Glucose came from Baker Chemical. Hexokinase was purified from yeast by R.H. Gooding according to the method of Schulze, Gazith and Gooding (1966). Arginine phosphate was synthesized enzymatically from ATP and arginine following the method of Marcus and Morrison (1964). Owing to its instability, it was converted to a barium salt for storage. Solutions of arginine phosphate were prepared by precipitating the barium with a calculated volume of 1N sodium sulfate. The precipitate was removed by centrifugation at 27,000 g for 1 hour.

3.2. Methods

3.2.1. Determination of enzyme activity

3.2.1.1. ATP synthesis: AP + ADP + H^+ → arginine + ATP

Arginine determination—The assay procedure was

based on the method of arginine determination described by Rosenberg, Ennor and Morrison (1956). In a typical assay, 0.45 ml of a stock solution containing AP, ADP, and $MgSO_4$ in Tris buffer, pH 7.2, was incubated at 30 C for 5 min. The reaction was started by adding 0.05 ml APK solution and was allowed to continue for 5 min. The final concentrations of AP, ADP, $MgSO_4$, and Tris were 5 mM, 1mM, 10 mM, and 50 mM, respectively. The reaction was stopped by adding 0.5 ml of a NaOH-EDTA solution containing concentrations of 0.08 N EDTA and 3 N NaOH. The color reaction with arginine was started by adding 1.0 ml of a developing solution followed by 5 ml distilled water, and the absorbance of the resulting solution was measured at 535 nm after 20 min. The developing solution was prepared by mixing 20 ml of 25% (w/v) α-napthol in n-propanol to 2.5 ml 1% (v/v) diacetyl in demineralized water; the mixture was diluted to 100 ml with n-propanol. The absorbance at 535 nm was converted to μmoles arginine present by using a standard curve. Controls were run in an identical way except that the NaOH-EDTA solution was added before adding the enzyme.

ATP determination - The method was modified from that of Morrison and James (1965). The reaction was started by adding 0.05 ml APK to 0.45 ml of an assay solution containing 5 mM AP, 1 mM ADP, and 10 mM Mg^{++}, 50 mM Tris, pH 7.2. The reaction was stopped after 10 min by adding 0.25 ml 30% acetic acid. The pH of the solution was brought to 7.9 by adding 0.2 ml 5N NaOH followed by 1.7 ml 50 mM Tris buffer pH 7.9. Then 0.2 ml 13.3 mM glucose and 0.2 ml 0.2 mM NADP were added. The solution was then transferred to a 3 ml cuvette in a thermospacer equilibrated to 30 C. Ten μl of a hexokinase solution were added and stirred. The absorbance at 340 nm was then set to read zero. Six μl of G-6-P DH were then added. The reading was taken when no further change in absorbance was

observed. The control was run in a similar way except that the acetic acid was added before adding the APK. By using a standard curve obtained with known amounts of ATP, absorbance at 340 nm is converted to μmoles ATP produced.

3.2.1.2. AP synthesis: arginine + ATP → AP + ADP + H^+

Enzyme activity was estimated by measuring the inorganic phosphate released after acid hydrolysis of arginine phosphate. The assay procedure was modified from that of Morrison, Griffiths and Ennor (1957). The reaction mixture contained a final concentration of 50 mM Tris, 5 mM ATP, and 10 mM each of arginine and $MgSO_4$, pH 8.3. In a typical assay, 0.9 ml of the stock solution was incubated at 30 C for 5 min. The reaction was started by adding 0.1 ml APK solution, and stopped after 5 min by adding 0.5 ml 30% acetic acid. The solution was placed in boiling water for exactly 1 min, after which it was immersed in an ice water bath. The color reaction with inorganic phosphate was started by adding 2.0 ml 5% (w/v) ammonium molybdate in 15% (v/v) H_2SO_4 in demineralized water followed half a minute later by 0.5 ml 0.25% (w/v) aminonaphthol sulfonic acid in demineralized water. The mixture was diluted with 5 ml demineralized water. The absorbance of the resulting blue solution was read at 540 nm after 20 min. Controls were run in the same way except that acetic acid was added before adding the enzyme. By using a standard curve established with known amounts of inorganic phosphate, A_{540} readings were converted to μmoles AP synthesized per min.

3.2.2. Protein determination

Protein concentrations were determined spectrophotometrically according to the method of Layne (1957).

4. EXPERIMENTAL RESULTS

4.1. Synthesis of arginine phosphate

Arginine phosphate was synthesized enzymatically from ATP and arginine, purified using a method similar to that of Marcus and Morrison (1964), and converted to the barium salt for storage.

A sample of the BaAP obtained was dissolved in demineralized water and hydrolyzed by immersing in boiling water for 1 min after the pH had been lowered to 1 by adding 1N HCl. The hydrolyzed BaAP together with unhydrolyzed BaAP and arginine were applied to a sheet of chromatographic paper. Descending chromatography was run for about 8 hours with a solvent containing n-propanol, ammonia, and water in a ratio of 60:30:10. The chromatogram was dried and sprayed with ninhydrin. The Rf values for arginine and hydrolyzed BaAP were 0.32, and that for unhydrolyzed BaAP was 0.075. The chromatogram is shown in Fig. 1. Determination of arginine and Pi showed that they constitute 39% and 28.6% of the sample, respectively. Elemental analysis by the microanalytical laboratory of the Chemistry Department gave the percentages of C, H, and N as 14.83, 4.02, and 12.29, respectively, compared to theoretical values of 18.0, 4.26, and 14.0 based on an assumed formula of $Ba(C_6H_{15}O_5N_4P)\ H_2O$.

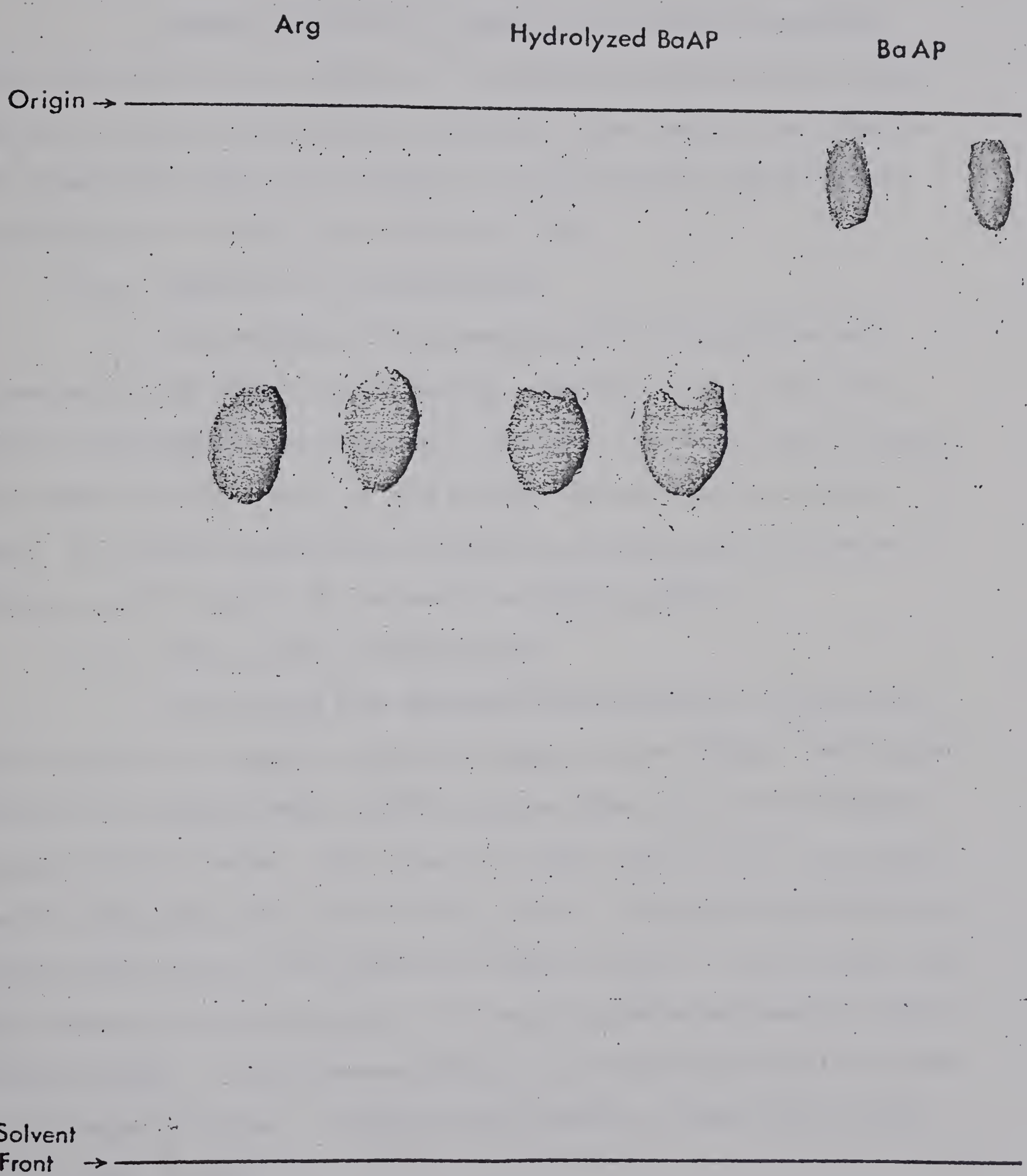
4.2. Purification of arginine phosphokinase

Purification procedure was carried out at 0-4 C. The buffer used was 10 mM Tris, 5 mM EDTA, pH 7.0, unless stated otherwise. APK activity was assayed in the direction of AP synthesis.

4.2.1. Extraction

Fifty grams of bee thoraces were homogenized for 3 min in 150 ml Tris buffer with an omni-mixer and centrifuged at 10,000 g for 30 min.

Fig. 1. Paper chromatogram of arginine, hydrolyzed BaAP, and unhydrolyzed BaAP. Solvent used was n-propanol, ammonia and water in a ratio of 60:30:10 in a descending chromatograph system. The chromatogram was developed with ninhydrin. The Rf value for arginine and hydrolyzed BaAP was 0.32 and for unhydrolyzed BaAP was 0.075.


Arg
Hydrolyzed BaAP
BaAP
Origin →
Solvent
Front →

The supernatant was saved. This procedure was repeated on the precipitate with 100 ml buffer and the supernatants were combined.

4.2.2. Ammonium sulfate fractionation

Granular $(NH_4)_2SO_4$ was added to the combined supernatant until 60% saturation was achieved. The precipitate formed after centrifugation at 10,000 g for 30 min was discarded. More $(NH_4)_2SO_4$ was added to the supernatant until 80% saturated and the precipitate collected after centrifugation at 10,000 g for 30 min was saved.

4.2.3. Sephadex G-100 chromatography

The precipitate from ammonium sulfate fractionation was dissolved in Tris buffer and put through a Sephadex G-100 column (1.4 x 102 cm) equilibrated with 50 mM Tris, 5 mM EDTA, 100 mM KCl, pH 7.0 buffer. The column was eluted with the same buffer. The fractions were about 2 ml each. The protein concentration estimated by measuring the absorbance at 280 nm, and APK activity of the eluate are shown in Fig. 2.

4.2.4. DEAE-cellulose chromatography

The solution from Sephadex chromatography was dialyzed overnight against two changes of about 50 volumes of Tris buffer. The dialyzed solution was pumped through a DEAE-cellulose column (2.5 x 24 cm) equilibrated with Tris buffer. The column was eluted with 0.1, 0.2, 0.4 M NaCl, and 30% $(NH_4)_2SO_4$ (w/v) in Tris buffer, pH 7.0. The protein concentration and the APK activity of the eluate are shown in Fig. 3. The fractions with most enzyme activity were pooled. The enzyme solution obtained was free of ATPase activity. A final concentration of 1 mM 2-mercaptoethanol was added to the enzyme solution. A summary of the data for a typical purification is presented in Table 1.

Fig. 2. Sephadex G-100 chromatography of 60-80% SAS fraction of honeybee thorax extract. The proteins were eluted with 50 mM Tris, 5 mM EDTA, 100 mM KCl, pH 7.0 buffer. Each fraction was about 2 ml.

-●- A_{280}

-o- v = μmoles AP/min

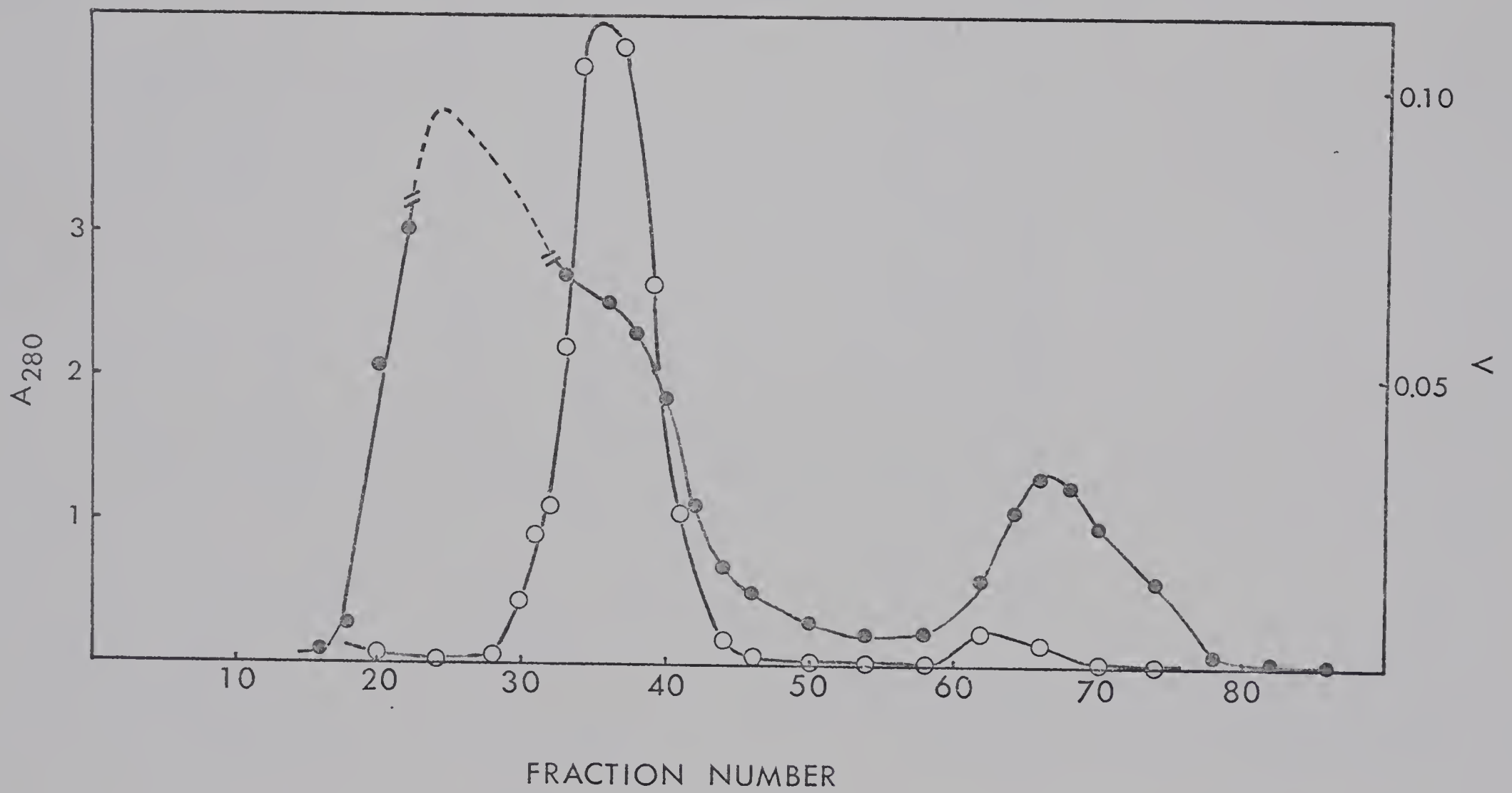

0.10
0.05
A
A_{280}
3
2
1
10
20
30
40
50
60
70
80
FRACTION NUMBER

Fig. 3. DEAE-cellulose chromatography of APK solution obtained from Sephadex G-100 chromatography. The column was eluted with 0.1, 0.2, 0.4 M NaCl, and 30% $(NH_4)_2SO_4$ in Tris buffer, pH 7.0.

1. 0.1 M NaCl
2. 0.2 M NaCl
3. 0.4 M NaCl
4. 30% $(NH_4)_2SO_4$

-●- A_{280}

-o- v = μmoles AP/min

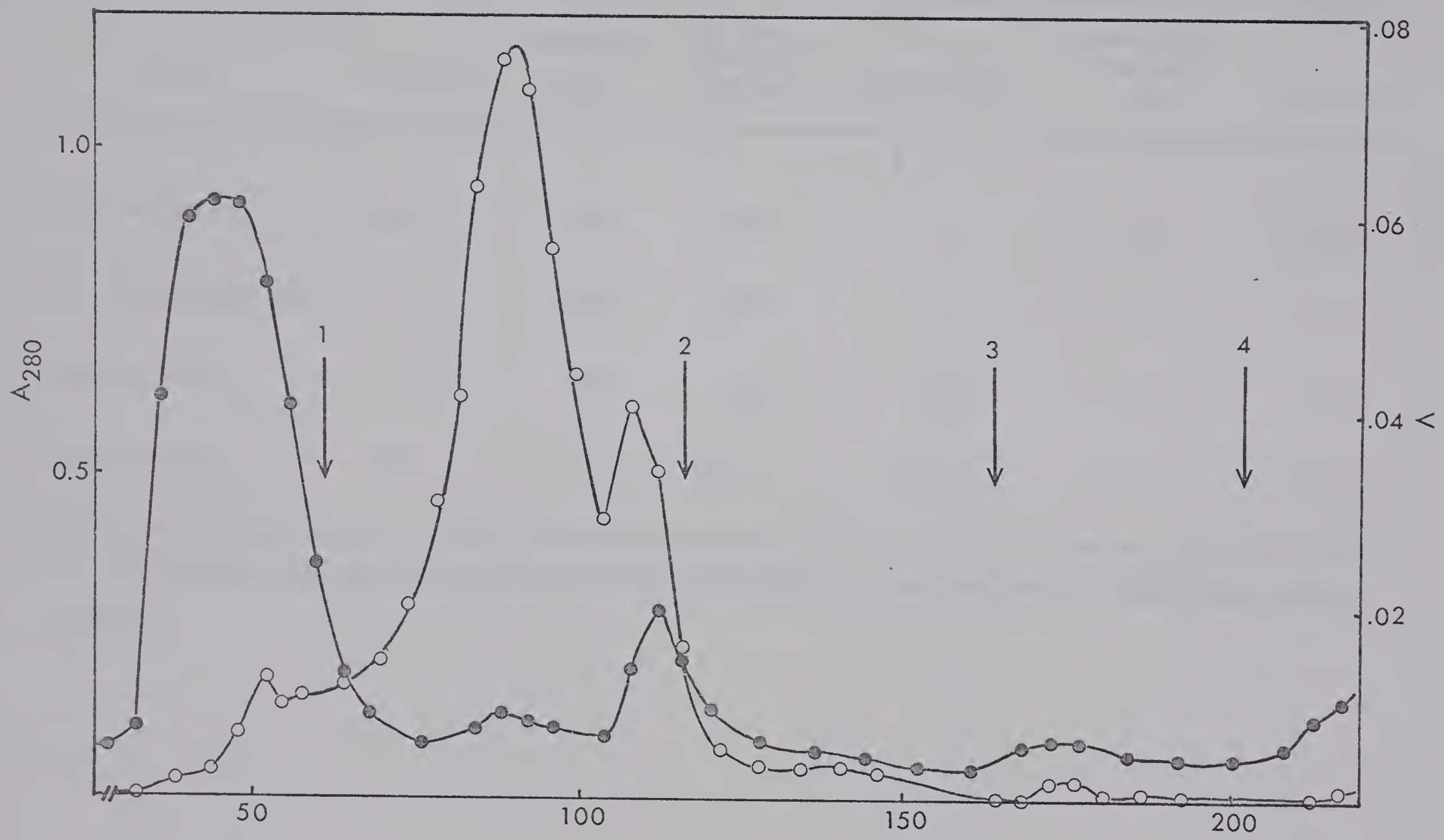

1.0
0.5
A_{280}
1
2
3
4
.08
.06
.04
.02
<
50
100
150
200
FRACTION NUMBER

Table 1. Purification of APK from honeybee thoraces.

Step	Vol.(ml)	Protein (mg)	Sp. Act. (μmoles/ min/mg)	Purification	Total Act. (μmoles/ min)	Yield (%)
Crude extract	222	910	0.29	1	264	100
Ppt. from 60-80% SAS	11.4	340	0.08*	--	--	--
Sephadex G-100	47.5	95	1.83	6.3	174	66
DEAE-cellulose	125	5	33.7	116	167	63

*The low specific activity was probably due to inhibition of the reaction by a high anion concentration.

4.3. Estimation of molecular weight by Sephadex G-100 gel chromatography

A Sephadex G-100 column (1.4 x 102 cm) was calibrated (method of Andrews, 1964), using γ-globulin, bovine albumin, haemoglobin, myoglobin, and cytochrome c. The molecular weight of the enzyme estimated by this method is 36,000 ± 3,000 with an assumed error in locating the enzyme peak of one fraction (Fig. 4).

4.4. Temperature stability of the enzyme

Assays were done in the direction of AP synthesis. Aliquots of the enzyme solution were incubated at various temperatures for 15 min and then assayed at 30 C for 15 min. Enzyme activity was constant at temperatures between 30 and 40 C but declined when incubated at 45 or 50 C (Fig. 5).

4.5. Effect of sulfhydryl compounds

Addition of 2-mercaptoethanol to the assay solution increased the enzyme activity, as determined in the direction of AP synthesis, by as much as 33% (Fig. 6). Final concentrations in the assay mixture of up to 0.05 mM cysteine and 0.4 mM reduced glutathion were also tested and a similar activating effect was observed.

4.6. Activation by bivalent cations

Enzyme activity was assayed in the direction of AP synthesis. The metal salts used were sulfates of Mg^{++}, Mn^{++}, and Cu^{++}, and chlorides of Co^{++} and Ca^{++}. No activity was detected without adding bivalent cations. Addition of 10 mM of Mg^{++}, Mn^{++}, Ca^{++}, and Co^{++} activated the enzyme to various extents. The enzyme was more active with Mn^{++} than with Mg^{++}; and Cu^{++}, Ca^{++}, or Co^{++} activated the enzyme to a much lesser extent. These results are presented in Table 2.

Fig. 4. Estimation of molecular weight by Sephadex G-100 gel chromatography. The proteins used and their molecular weights were:

1.	γ-globulin	160,000
2.	bovine albumin (dimer)	134,000
3.	bovine albumin (monomer)	67,000
4.	haemoglobin	64,500
5.	myoglobin	17,800
6.	cytochrome c	12,400

The molecular weight of APK estimated by this method is 36,000 ± 3,000.

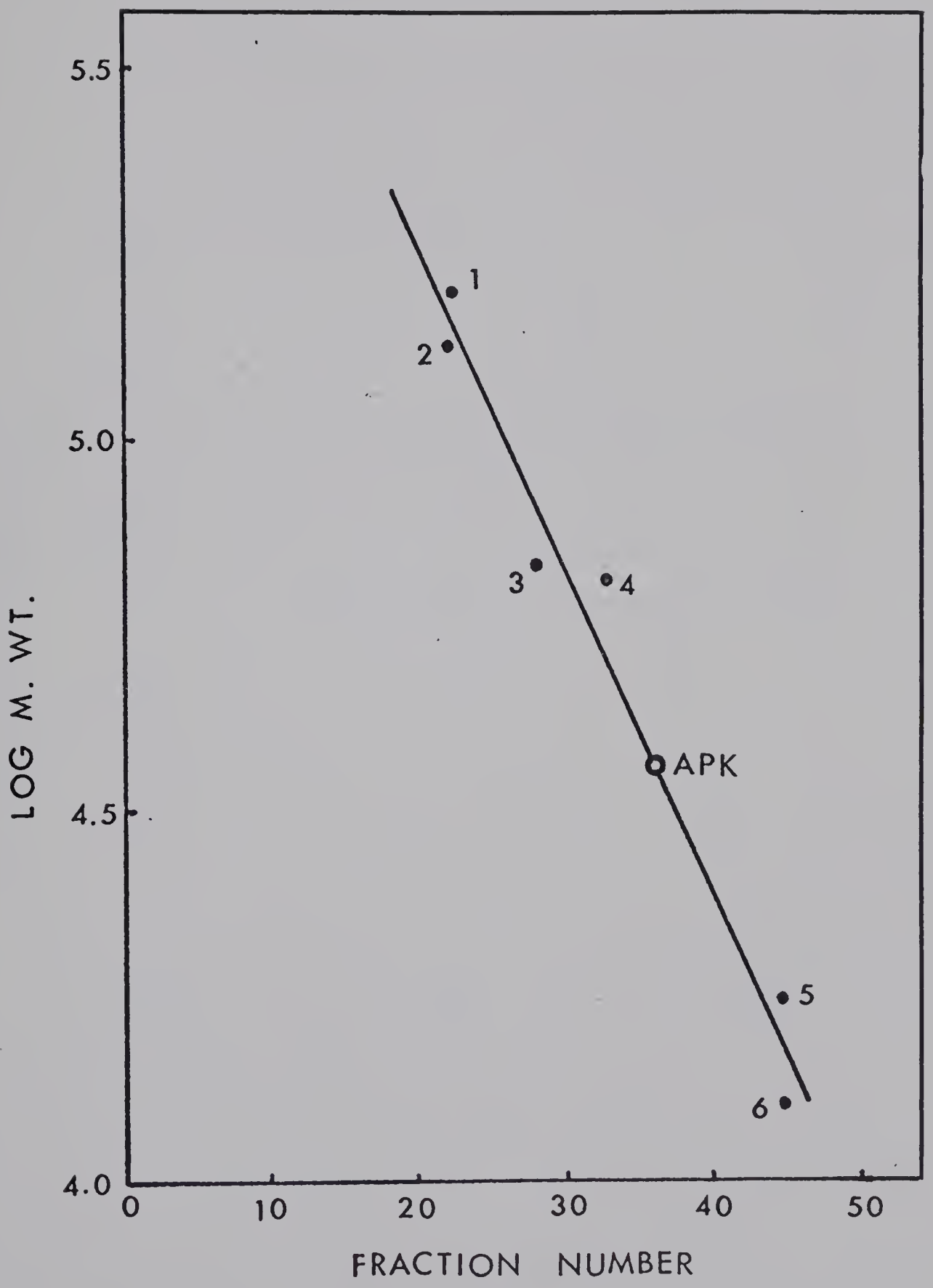

5.5
5.0
4.5
4.0
LOG M. WT.
1
2
3
4
APK
5
6
0
10
20
30
40
50
FRACTION NUMBER

Fig. 5. Temperature stability of APK. Aliquots of enzyme solution were incubated at various temperatures for 15 min and then assayed at 30 C for 15 min. The activity of the enzyme incubated at 30 C was taken as 100%.

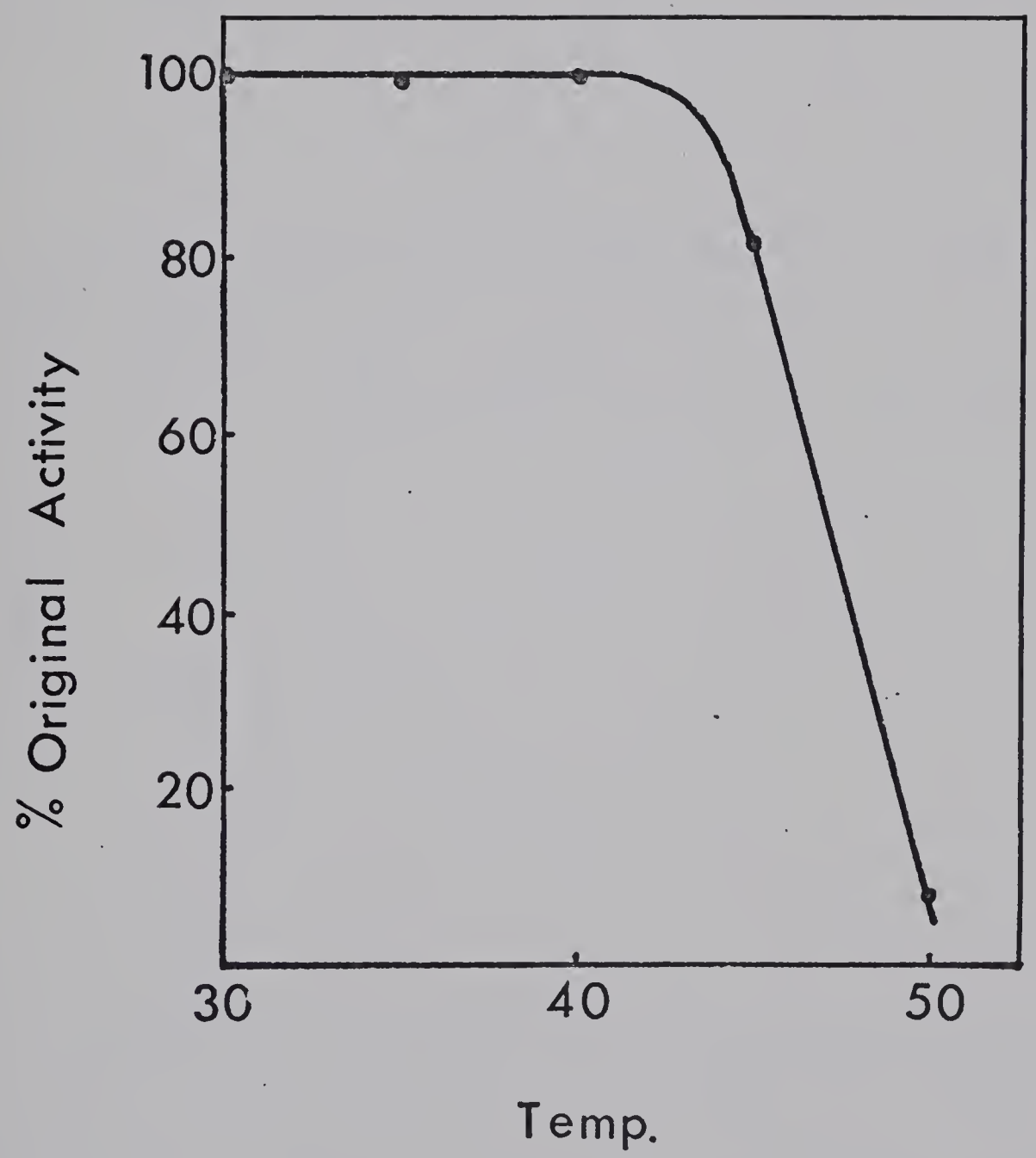

100
80
60
40
20
% Original Activity
30
40
50
Temp.

Fig. 6. Effect of 2-mercaptoethanol on APK activity. Addition of 2-mercaptoethanol to the reaction solution increased the enzyme activity by as much as 33%.

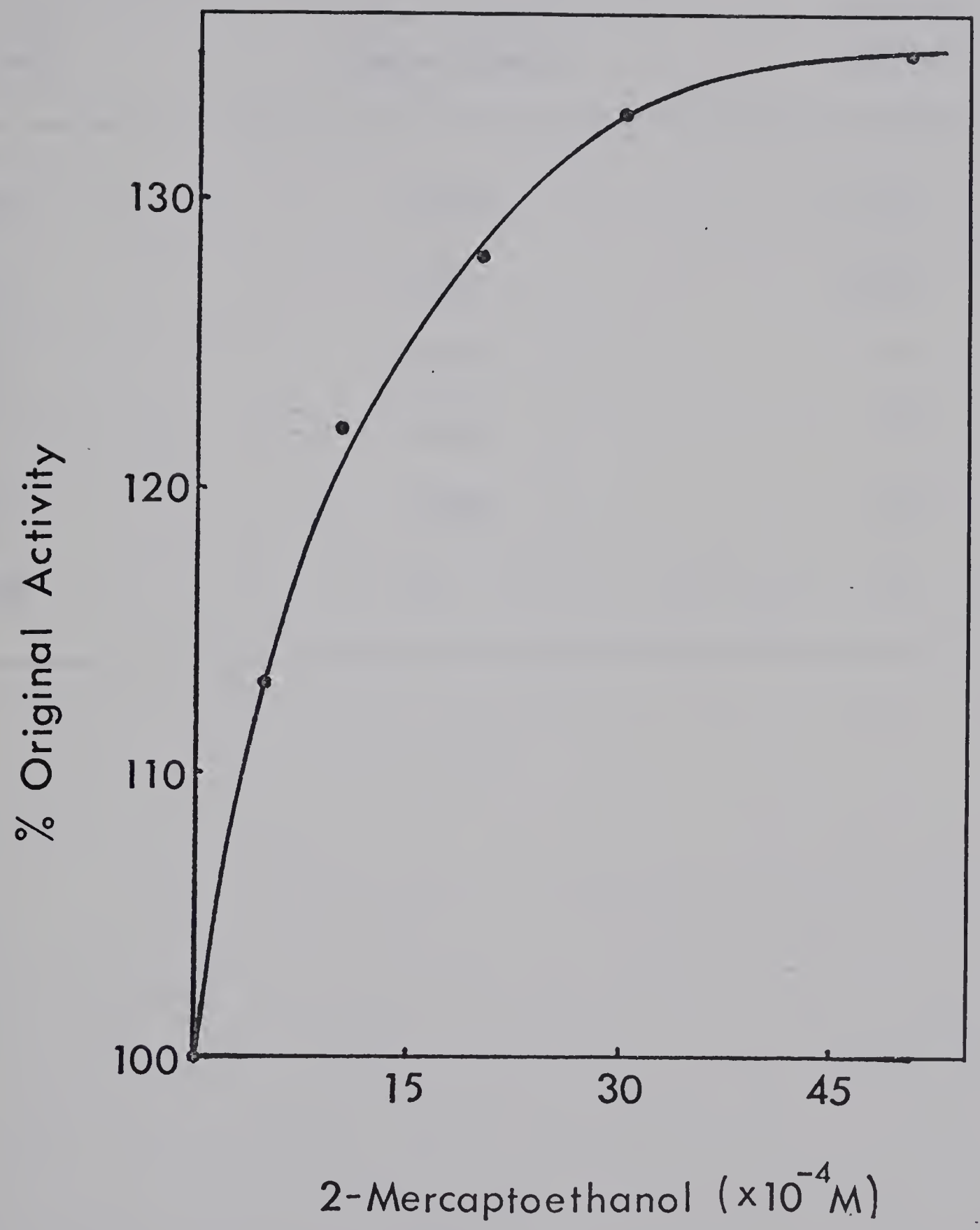

% Original Activity
130
120
110
100
15
30
45
2-Mercaptoethanol (x10⁻⁴M)

Table 2. Activation of APK by metal ions.

Metal ion (10 mM)	Enzyme activity (μmoles AP/min)	Percentage activity
Mn^{++}	0.082	111
Mg^{++}	0.074	(100)
Ca^{++}	0.017	23
Co^{++}	0.010	13.5
Cu^{++}	0.006	8.1
None	0	0

4.7. Specificity of arginine phosphokinase

When ATP was replaced by the same concentration of UTP, CTP, GTP, or ADP, no activity was observed.

The ability of the enzyme to phosphorylate several guanidino compounds was determined. The final concentration of the guanidines in the reaction mixture was 10 mM. The results of the experiment are shown in Table 3. The enzyme phosphorylated L-arginine methyl ester and D-arginine to a limited extent but did not phosphorylate guanidoacetic acid, hippuryl-L-arginine, nor creatine.

4.8. Effect of pH on APK activity

4.8.1. ATP synthesis

The assays were done in 50 mM Tris buffer between pH 7.0 and 9.5, and in 100 mM bicarbonate, 50 mM Tris buffer between pH 5.0 and 7.7. To adjust the pH, 1 N NaOH or 1 N HCl was used. APK used was 0.4 µg in a reaction volume of 0.5 ml. Reaction time was 5 min. Maximum activity was observed around pH 7.1-7.2 (Fig. 7).

4.8.2. AP synthesis

The assays were done in 100 mM bicarbonate and 50 mM Tris buffer; pH adjustments were made by adding 1 N of either NaOH or HCl. Enzyme used was 1.6 µg in a reaction volume of 1.0 ml. Reaction time was 15 min. Optimum activity occurred around pH 8.3. At pH's higher than 9.0, the enzyme activity declined rapidly (Fig. 8).

4.9. Effect of temperature on velocity

4.9.1. ATP synthesis

The assay solution contained a final concentration of 5 mM AP, 1 mM ADP, and 10 mM Mg^{++} in 50 mM Tris buffer, pH 7.2. The assay

Table 3. Specificity of APK: phosphorylation of guanidines.

Guanidines (10 mM)	Enzyme activity (μmoles AP/min)	Percentage activity
L-arginine	0.13	(100)
L-arginine methyl ester	0.056	43
D-arginine	0.016	12.3
Guanidoacetic acid	0	0
Hippuryl-L-arginine	0	0
Creatine	0	0

Fig. 7. Effect of pH on the velocity of ATP synthesis. The assays were done in 50 mM Tris, 100 mM bicarbonate buffer between pH 5.0 and 7.7 (-●-), and in 50 mM Tris between pH 7.0 and 9.5 (-o-). Either 1N NaOH or 1N HCl was used in adjusting the pH. Maximum activity occurred around pH 7.2.

v = μmoles ATP/min.

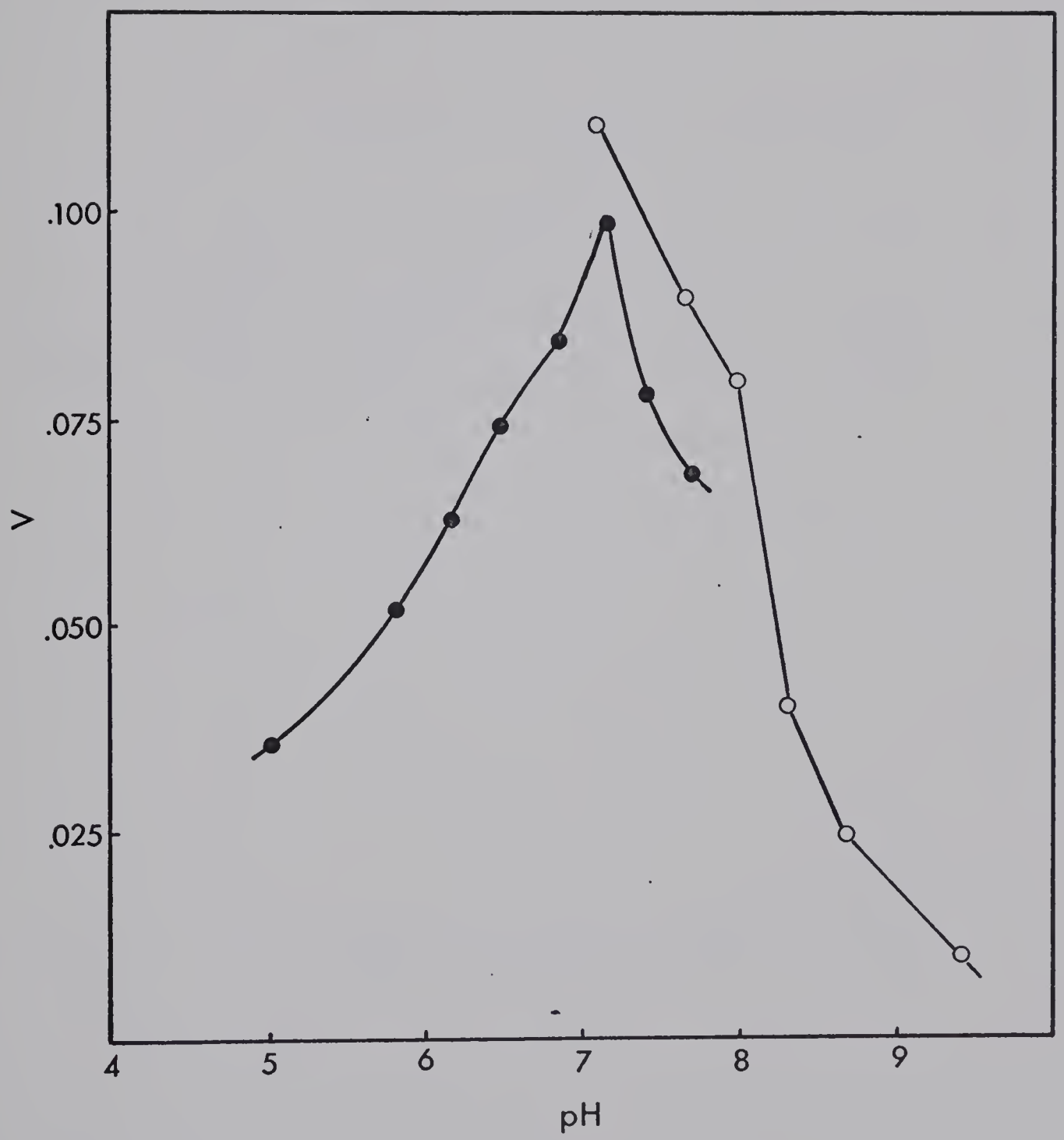
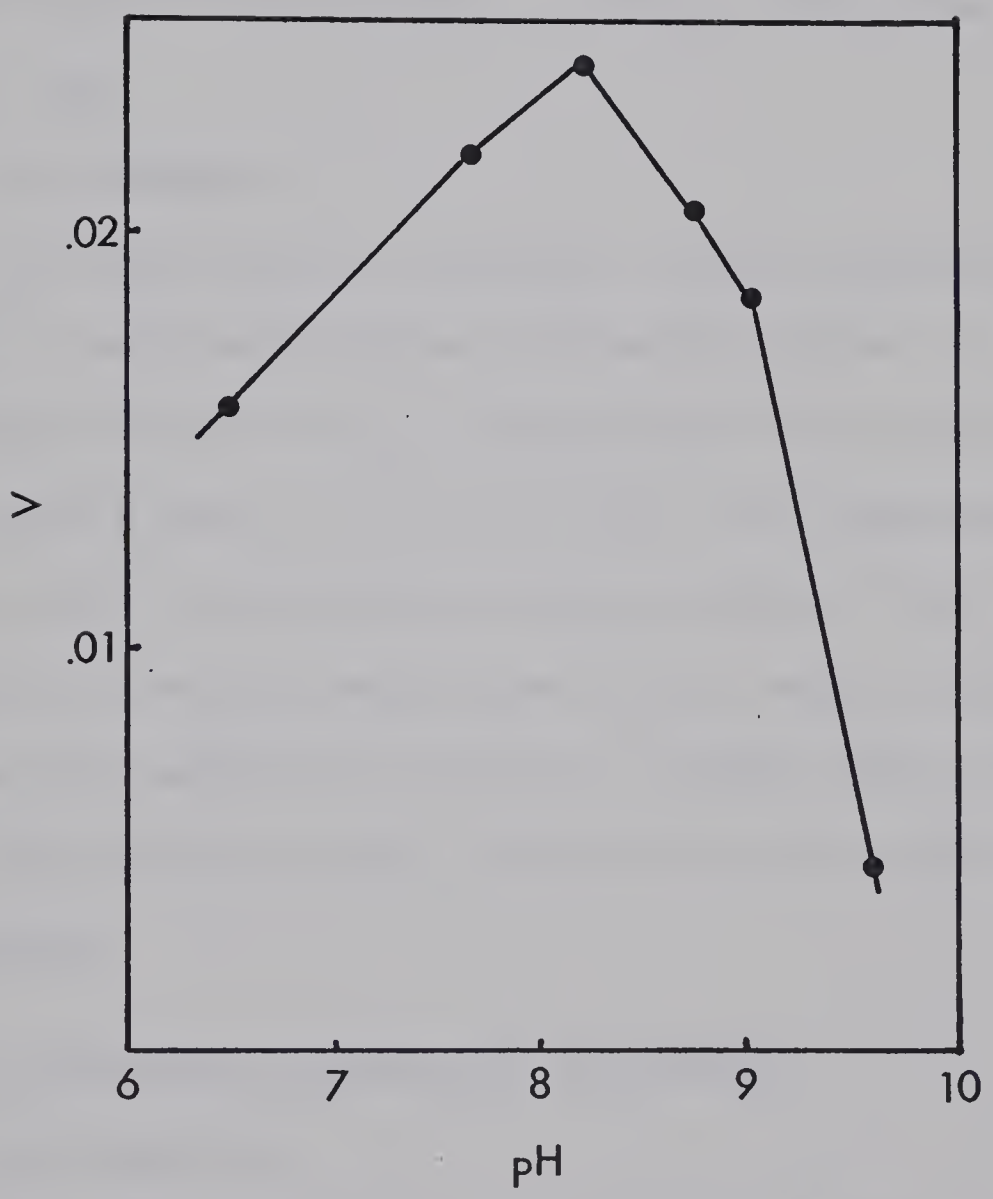

.100
.075
.050
.025
V
4
5
6
7
8
9
pH

Fig. 8. Effect of pH on the velocity of AP synthesis. The assays were done in 50 ml Tris, 100 mM bicarbonate buffer. Either 1N NaOH or 1N HCl was used in adjusting the pH. Optimum activity occurred around pH 8.3.

v = μmoles AP/min.


.02
.01
V
6
7
8
9
10
pH

solution was first incubated at the assay temperature for 5 min. The reaction was started by adding 0.2 µg APK to the assay solution. Reaction time was 5 min for assays at 18, 23, 25, and 29.5 C, 2 min for assays at 32, 35, 37.5, and 41 C, and 1 min for assays at 44, 50, and 55 C. The velocities of the reaction at these temperatures are shown in Fig. 9A. When the reciprocals of absolute temperatures for the range of 18-44 C were plotted against the logarithm of velocity, a straight line was obtained. The activation energy calculated from the slope of this line was 10,500 cal/mole (Fig. 9B).

4.9.2. AP synthesis

The assay solution contained a final concentration of 5 mM ATP, 10 mM Mg^{++}, and 10 mM arginine in 50 mM Tris buffer pH 8.3. The amount of enzyme used was 1.6 µg. The duration of the assays for 22, 29, 34, 39, 45, and 50 C were 5, 5, 3, 2, 1, and 1 min, respectively. Velocity increased from 22-45 C; beyond this, velocity declined (Fig. 10A). When the reciprocals of absolute temperatures for the range of 22-39 C were plotted against the logarithm of velocity a straight line was obtained (Fig. 10B). The activation energy calculated from the slope of the graph was 7,500 cal/mole.

4.10. Effect of magnesium concentration on velocity

4.10.1. ATP synthesis

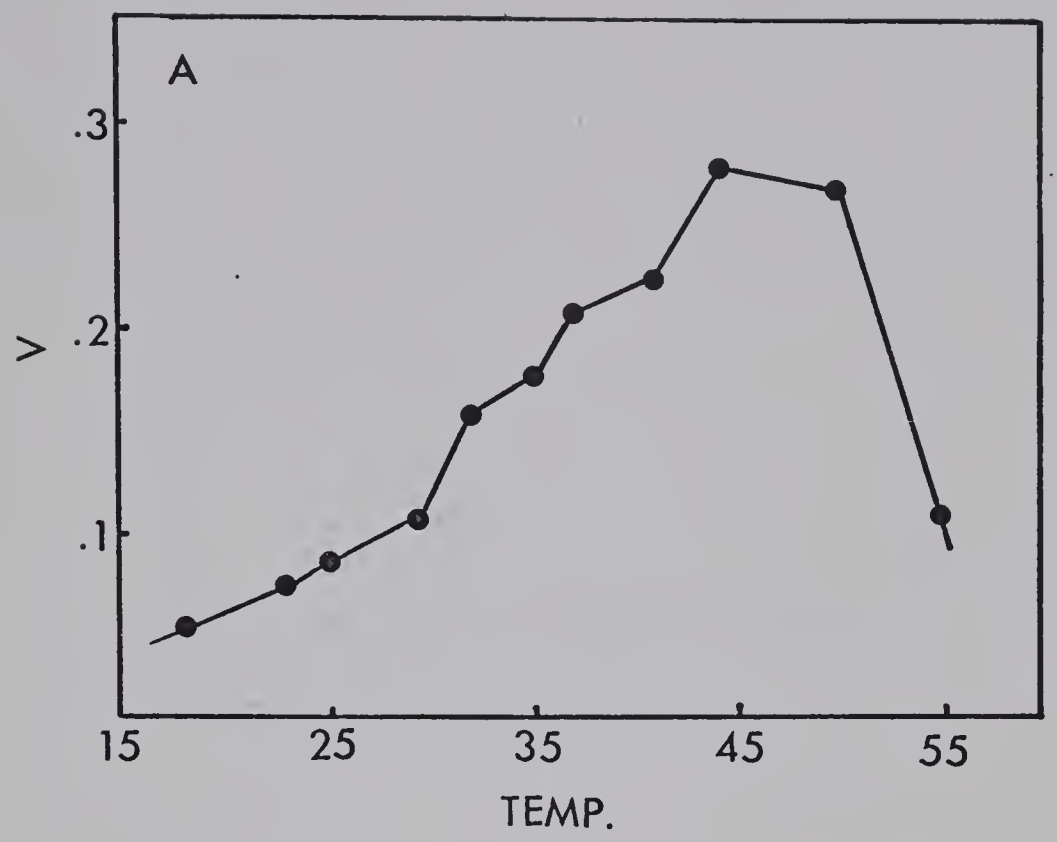
The effects of Mg^{++} concentration at 0.5 mM and 1.0 mM ADP concentrations are shown graphically in Fig. 11. The AP concentration in the reaction mixture was 5 mM and 0.2 µg of APK/assay was used. A trace of activity was observed without Mg^{++}. The activity increased sharply when Mg^{++} was added. The optimum ratio of Mg^{++}:ADP was about 4:1.

Fig. 9A. Effect of temperature on the velocity of ATP synthesis.

Fig. 9B. The Arrhenius plot of the data from Fig. 9A. The activation energy calculated from the slope of the curve was 10,500 cal/mole.

T = absolute temperature

v = μmoles ATP/min.


A
.3
.2
.1
V
15
25
35
45
55
TEMP.

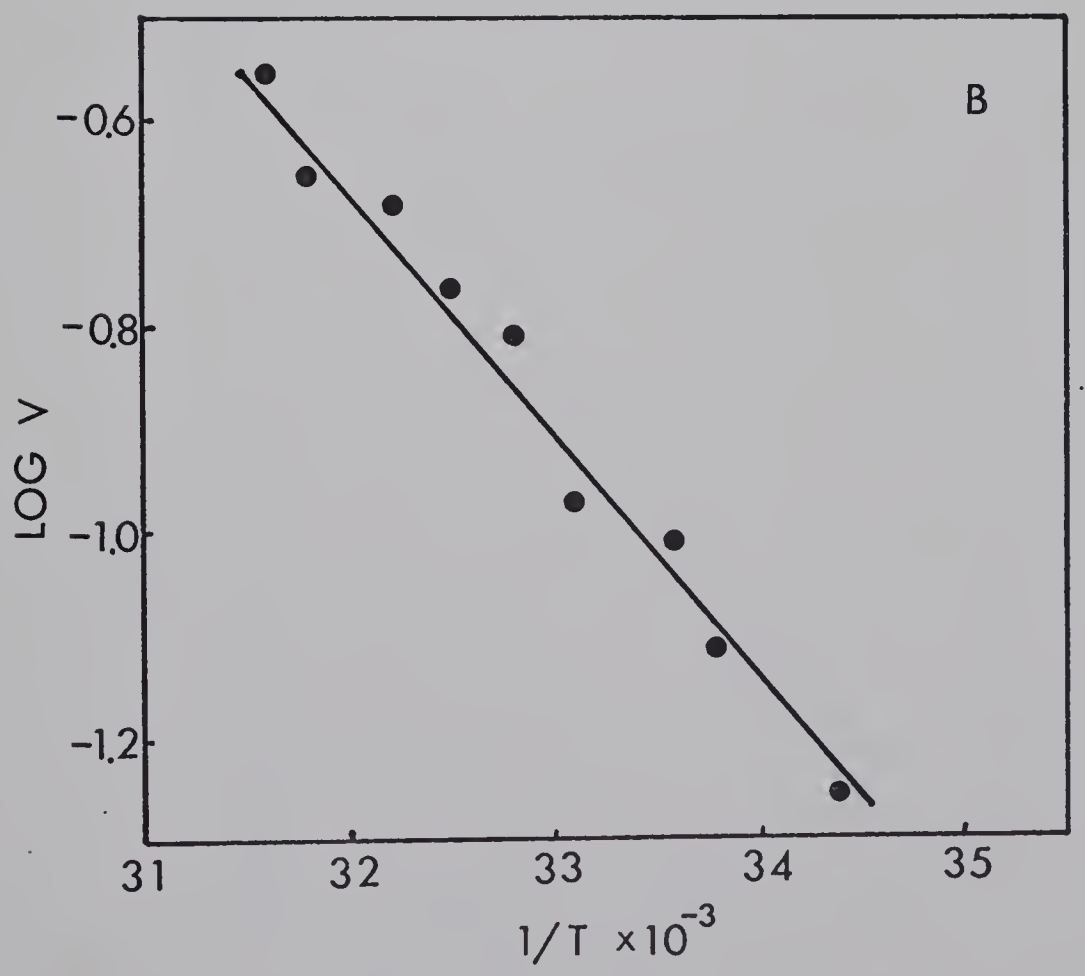
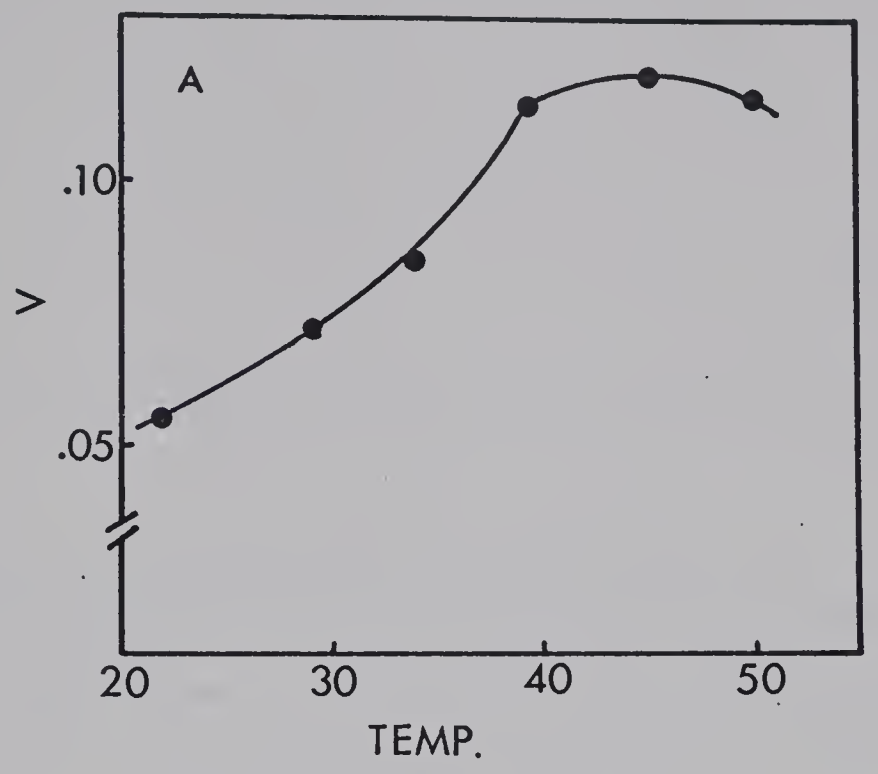

B
-0.6
-0.8
-1.0
-1.2
LOG V
31
32
33
34
35
1/T ×10⁻³

Fig. 10A. Effect of temperature on the velocity of AP synthesis.

Fig. 10B. The Arrhenius plot of the data from Fig. 10A. The activation energy calculated from the slope of the plot was 7,500 cal/mole.

T = absolute temperature

v = μmoles AP/min.


A
.10
.05
V
20
30
40
50
TEMP.

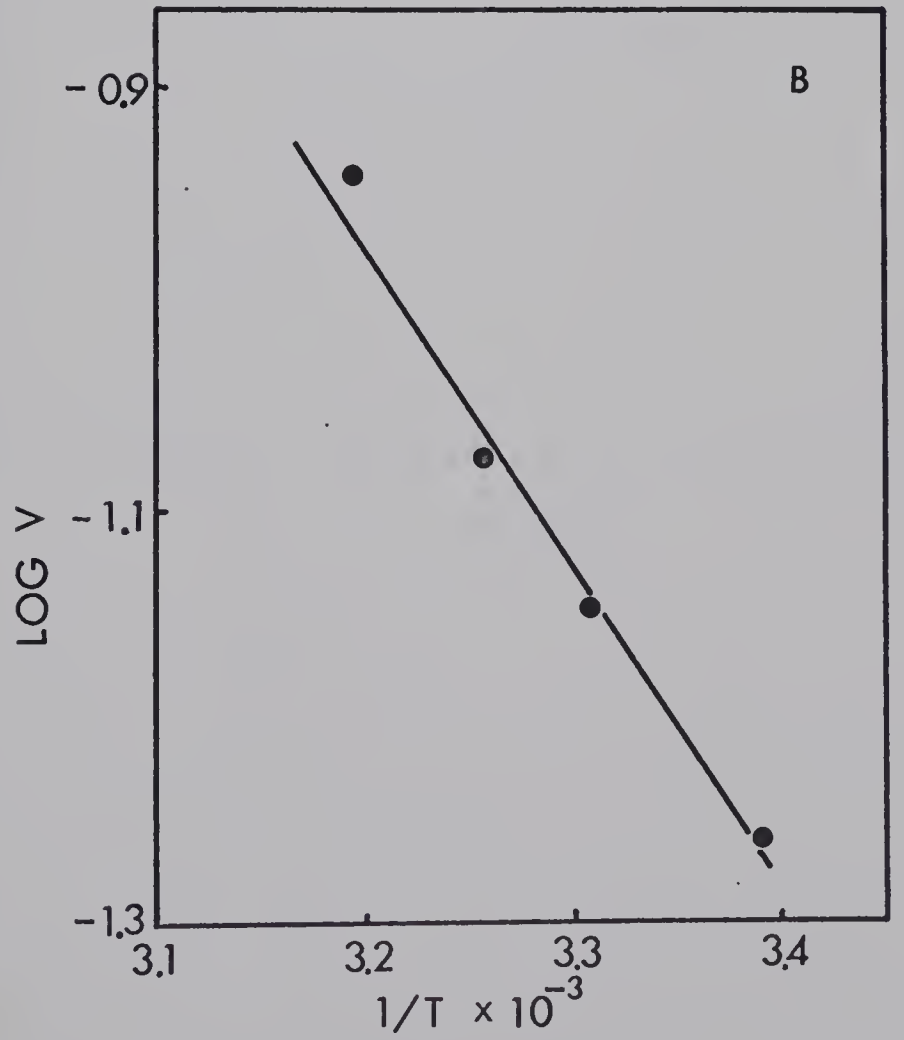
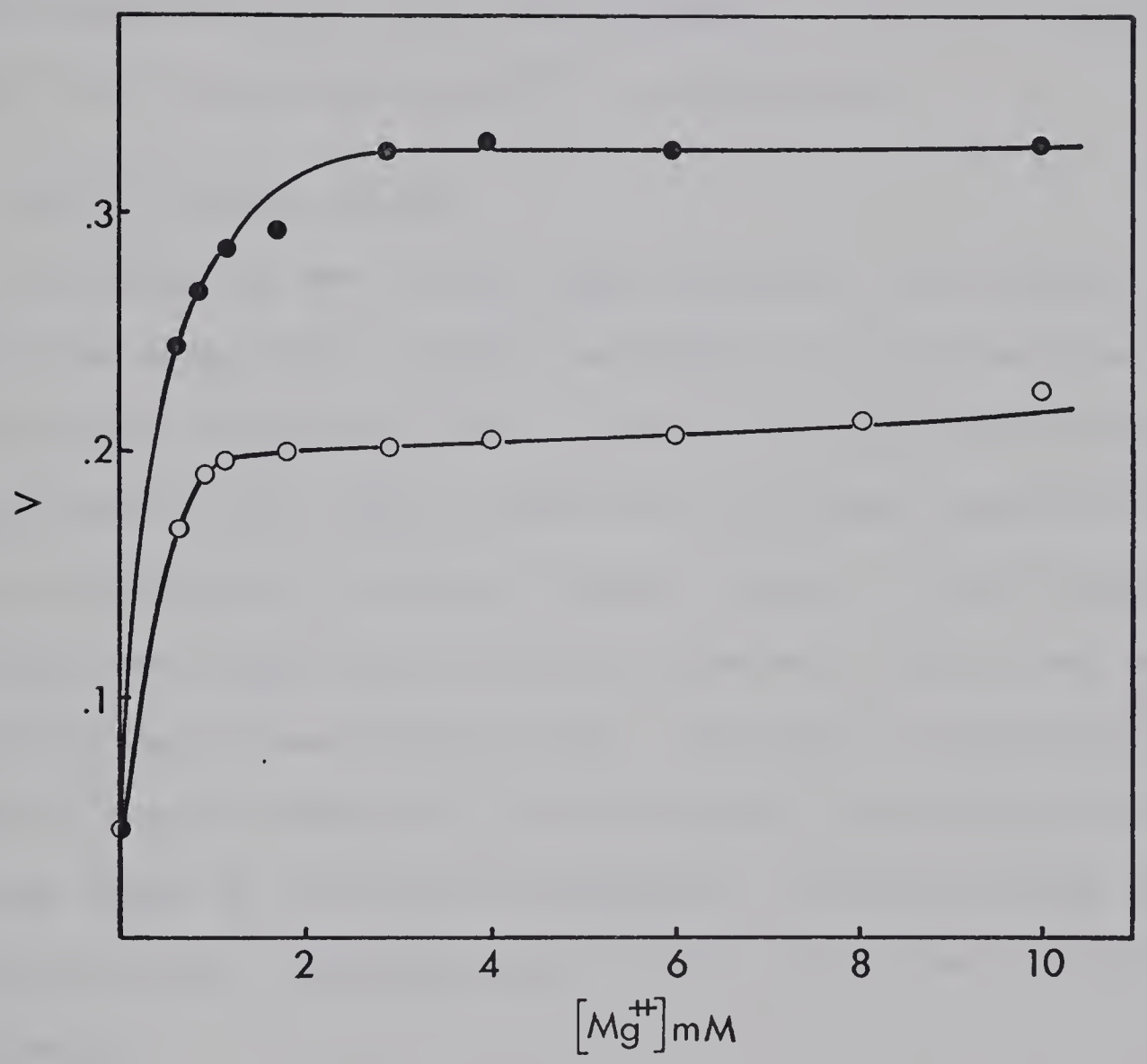

B
-0.9
-1.1
-1.3
LOG V
3.1
3.2
3.3
3.4
1/T x 10⁻³

Fig. 11. Effect of magnesium concentration on the velocity of ATP synthesis. The reaction mixture contained 5 mM AP. The concentrations of ADP were:

-o- 0.5 mM ADP

-●- 1.0 mM ADP

v = μmoles ATP/min.


.3
.2
.1
V
2
4
6
8
10
[Mg++]mM

4.10.2. AP synthesis

The effects of Mg^{++} concentration on velocity was studied with three ATP concentrations (Fig. 12). The concentration of arginine was 10 mM. The maximum velocity was reached when the molar ratio of Mg^{++}: ATP was 1:1. Increase in the ratio caused a slight decline in velocity. Similar results were obtained with Mn^{++}. The Lineweaver-Burk plot of the reaction velocities against ATP concentrations at 5 mM and 10 mM Mg^{++} indicates that the inhibition by Mg^{++} was competitive.

4.11. Initial velocity studies

To ensure that only initial linear velocities were measured, the reaction time within which velocity was linear was determined prior to the initial velocity experiments. This was done by measuring the velocity at increasing assay time, using the lowest and the highest substrate concentrations that were used in initial velocity studies. For ATP synthesis the velocity was linear when the reaction time was 10 min or less and for AP synthesis when it was 15 min or less. The amount of enzyme used was 0.2 µg in a reaction volume of 0.5 ml for the ATP synthesis and 1.6 µg in a reaction volume of 1.0 ml for AP synthesis. The reaction times used in the initial velocity experiments were 5 min for ATP synthesis and 10 min for AP synthesis.

4.11.1. ATP synthesis

4.11.1.1. Varying ADP concentrations at fixed AP concentrations

The reaction mixture contained varied concentrations of ADP, and a concentration of Mg^{++} which was 10 times that of ADP, in 50 mM Tris buffer, pH 7.2. AP concentrations were 5 mM, 3.5 mM, 2 mM, and 1 mM. Fig. 13 shows double reciprocal plots of the data. Changing the

Fig. 12. Effect of Mg^{++} concentration on the velocity of AP synthesis. The concentration of arginine was 10 mM. The concentrations of ATP were:

-●- 2.5 mM ATP

-▲- 3.75 mM ATP

-■- 5.0 mM ATP

v = μmoles AP/min.

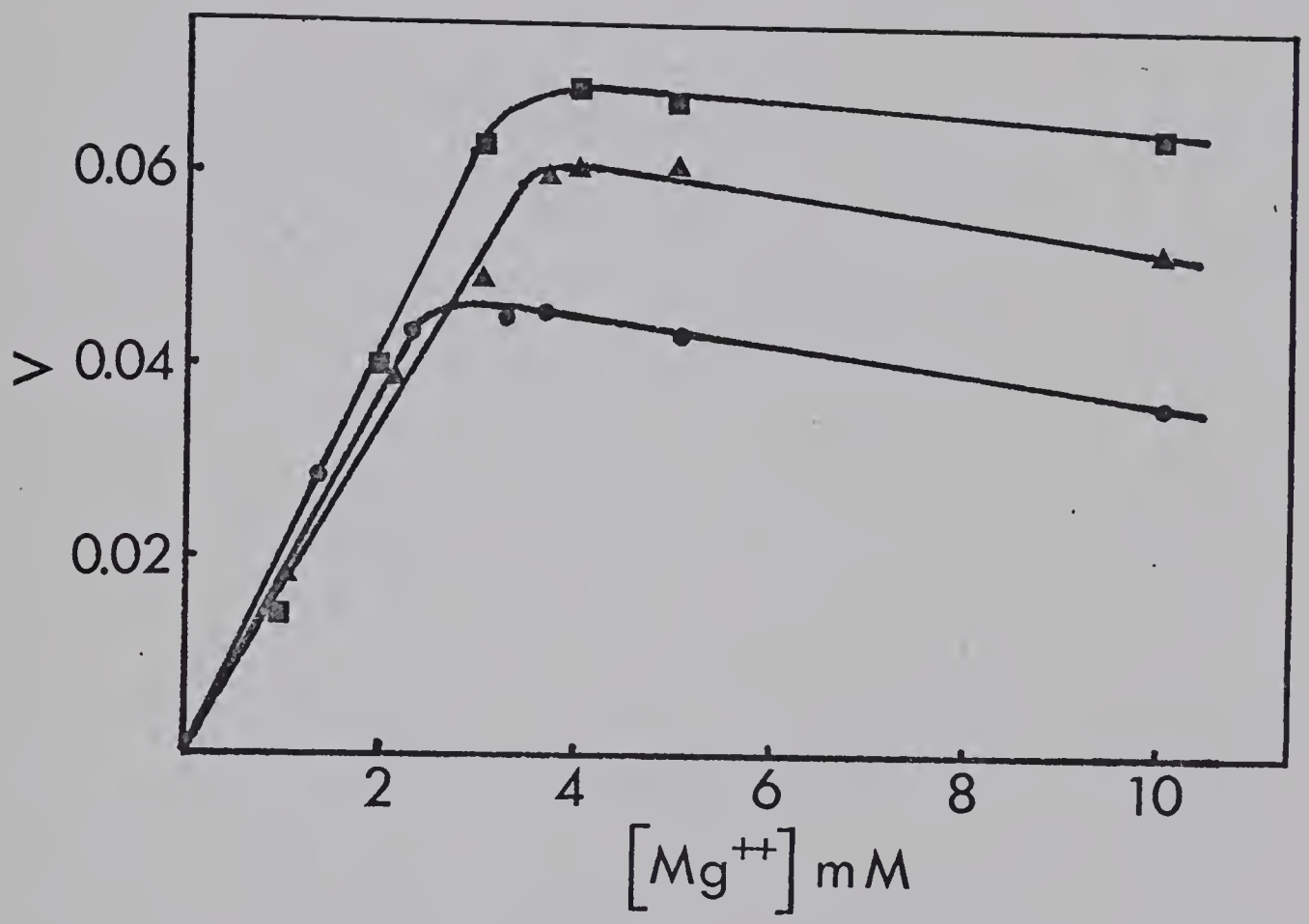

0.06
> 0.04
0.02
2
4
6
8
10
[Mg++] mM

Fig. 13. Initial velocity studies in the direction of ATP synthesis with ADP as the varied substrate. The Mg^{++} concentration was twice that of ADP concentration. The concentrations of AP were:

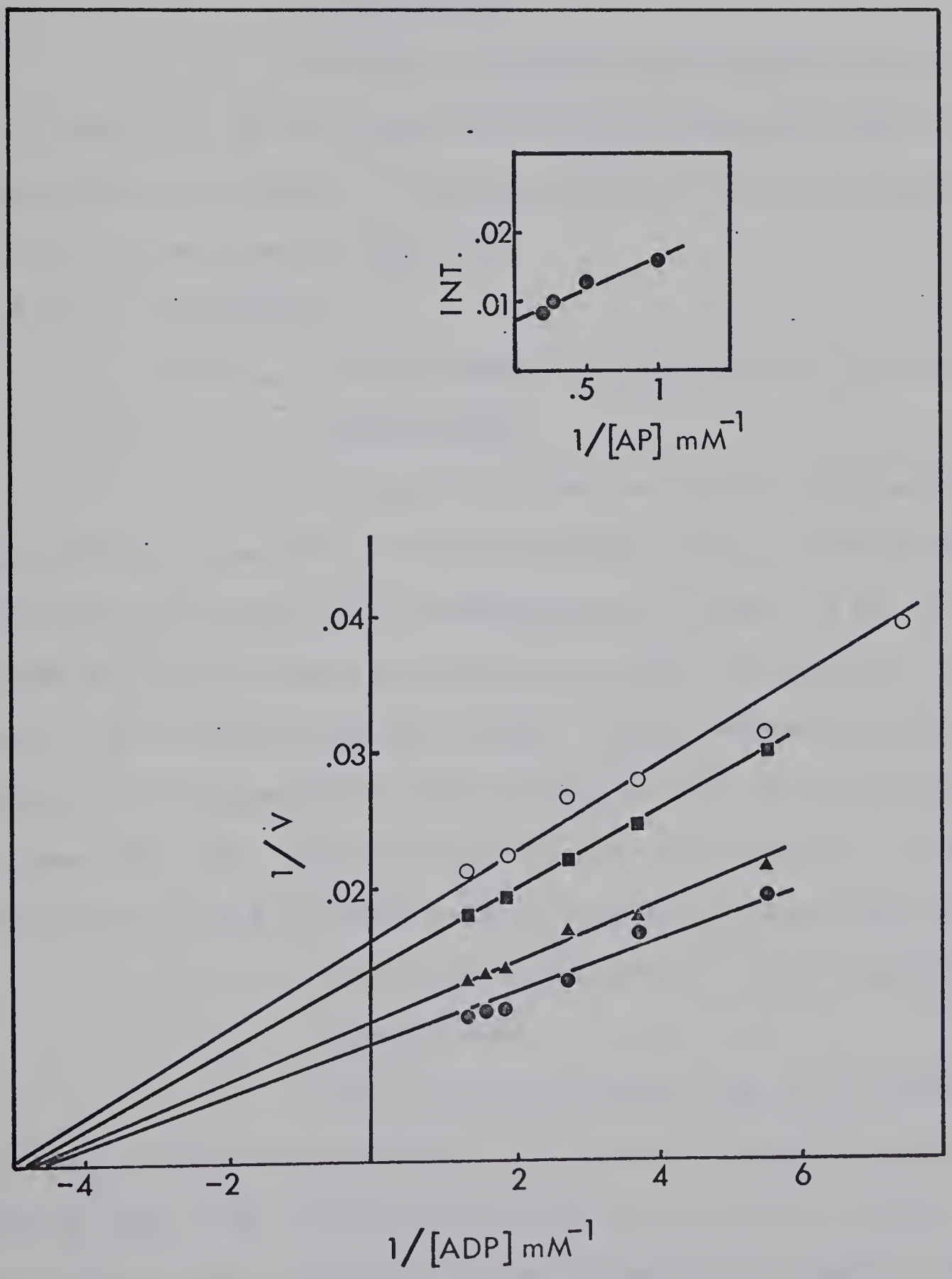
-●- 5.0 mM

-▲- 3.5 mM

-■- 2.0 mM

-o- 1.0 mM

v = µmoles ATP/min/mg protein.


INT.
.02
.01
.5
1
1/[AP] mM^{-1}
1/v
.04
.03
.02
-4
-2
2
4
6
1/[ADP] mM^{-1}

AP concentration changed both the slope of the line and its Y-axis intercept. The lines intersect left of the Y-axis.

4.11.1.2. Varying AP concentrations at fixed ADP concentrations

The assay conditions were the same as in section 4.11.1.1, except that AP was varied while the ADP concentration was one of 0.45 mM, 0.27 mM, or 0.079 mM. A similar pattern of lines as discussed in section 4.11.1.1 was observed (Fig. 14).

4.11.2. AP synthesis

4.11.2.1. Varying arginine concentrations at fixed ATP concentrations

The reaction volume was 1.0 ml and it contained arginine, ATP, Mg^{++}, and APK in 50 mM Tris buffer, pH 7.2. The concentration of arginine was varied, ATP concentration was 5.0 mM, 2.0 mM, 1.25 mM, or 0.75 mM, Mg^{++} concentration was twice that of ATP, the amount of APK used was 1.6 µg. Reaction time was 10 min. Double reciprocal plots show that change in ATP concentration affects both the slope and the intercept of the lines (Fig. 15). The lines intersect left of the Y-axis. Data from experiments done at pH 8.3 showed a similar pattern of intersecting lines.

4.11.2.2. Varying ATP concentrations at fixed arginine concentrations

Assay conditions were the same as in section 4.11.2.1 except that ATP concentration was varied and arginine concentration was fixed at 5 mM, 2 mM, 1.25 mM, or 0.75 mM. As in 4.11.2.1, double reciprocal plots of the data give a series of lines which intersect left of the Y-axis (Fig. 16). A similar pattern of lines was obtained when the

Fig. 14. Initial velocity studies for ATP synthesis with AP as the varied substrate. Mg^{++} concentration was ten times that of ADP. The concentrations of ADP were:

-●- 0.45 mM

-▲- 0.27 mM

-■- 0.124 mM

-o- 0.079 mM

v = µmoles ATP/min/mg protein.

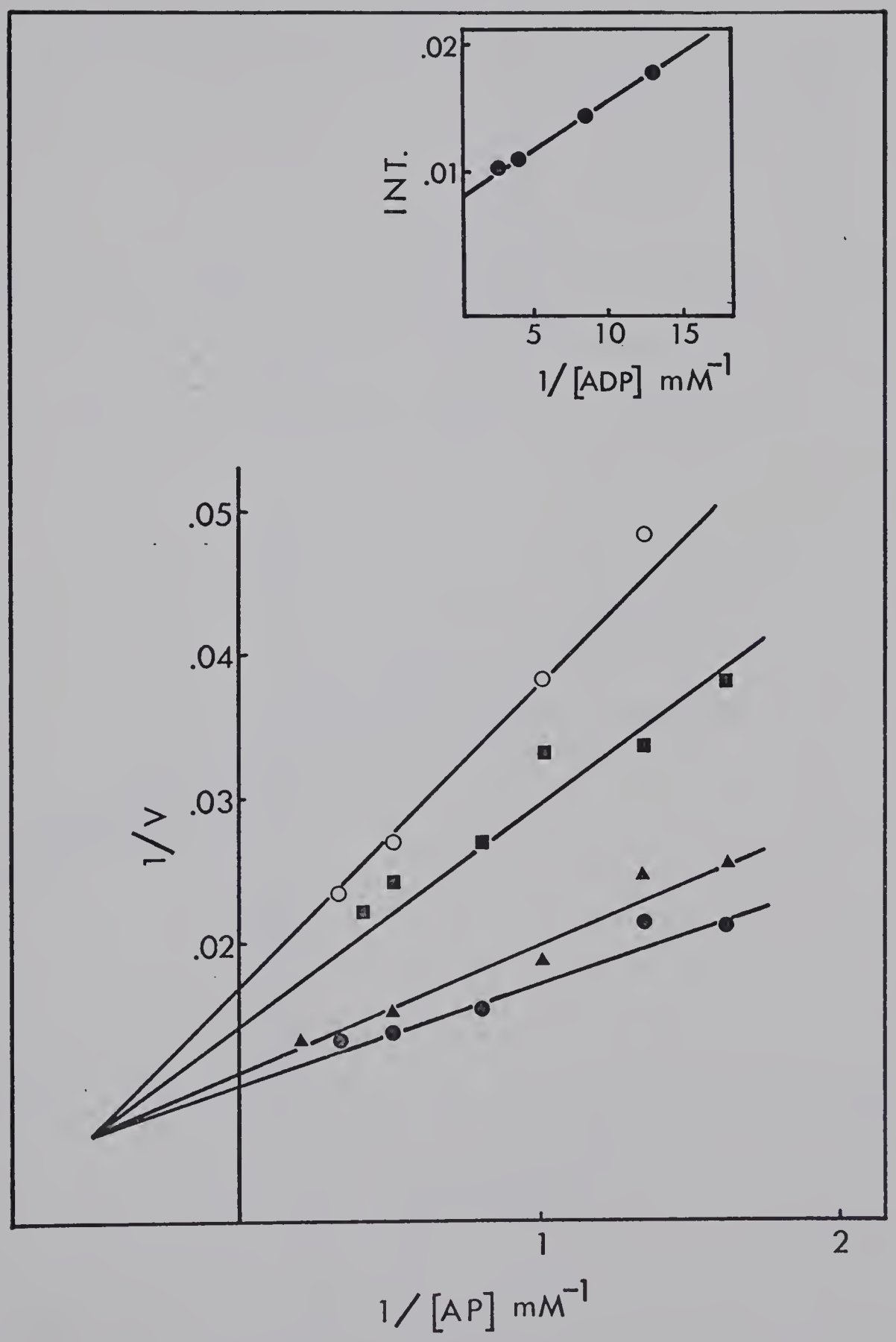

.02
.01
INT.
5
10
15
1/[ADP] mM⁻¹
.05
.04
.03
.02
1/v
1
2
1/[AP] mM⁻¹

Fig. 15. Initial velocity studies for AP synthesis with arginine as the varied substrate. Mg^{++} concentration was twice that of ATP. The concentrations of ATP were:

-●- 5.0 mM

-▲- 2.0 mM

-■- 1.25 mM

-o- 0.75 mM

v = µmoles AP/min/mg protein.

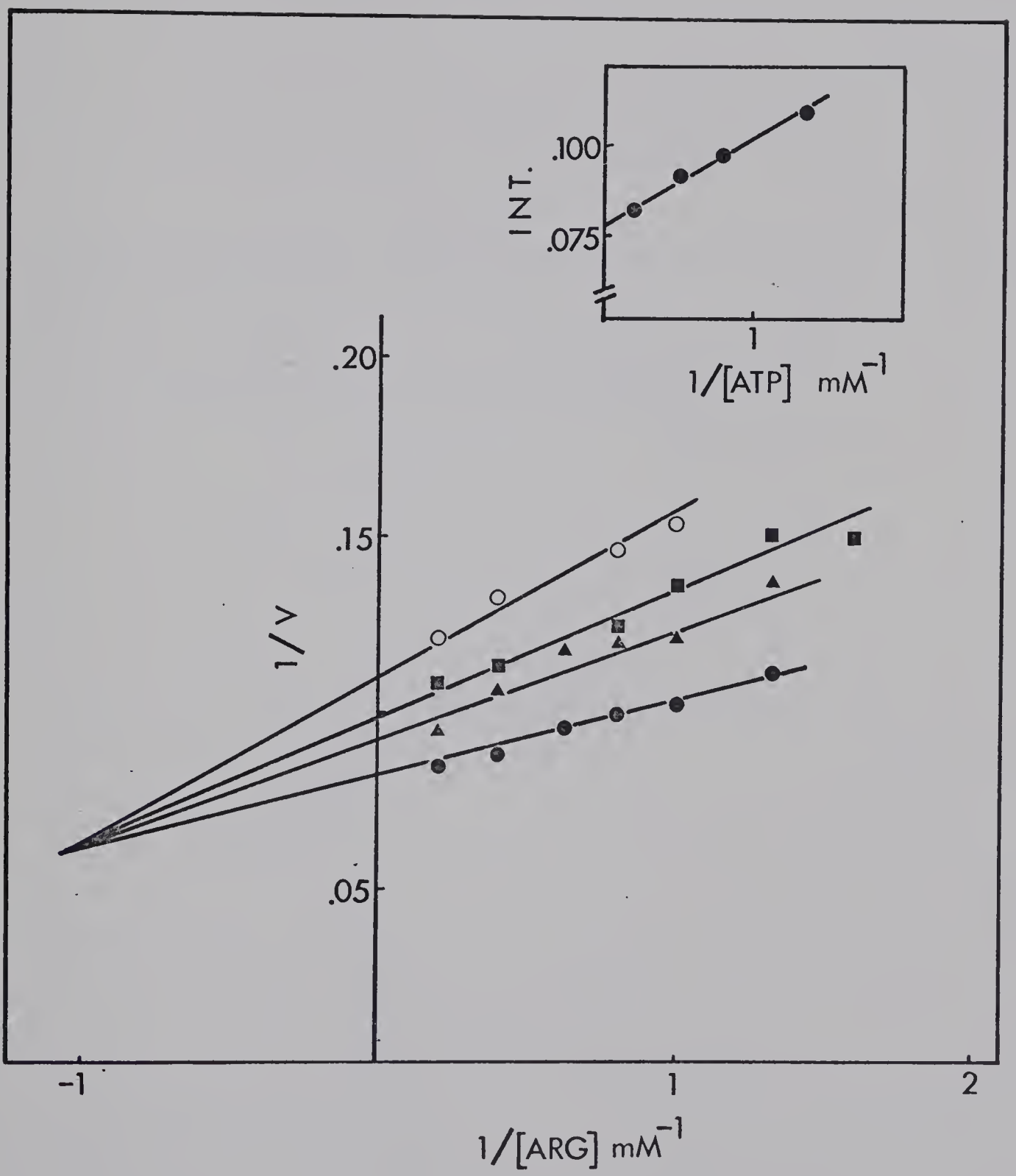

INT.
.100
.075
1
1/[ATP] mM^{-1}
.20
.15
.05
1/V
-1
1
2
1/[ARG] mM^{-1}

Fig. 16. Initial velocity studies for AP synthesis with ATP as the varied substrate. Mg^{++} concentration was twice that of ATP. The concentrations of arginine were:

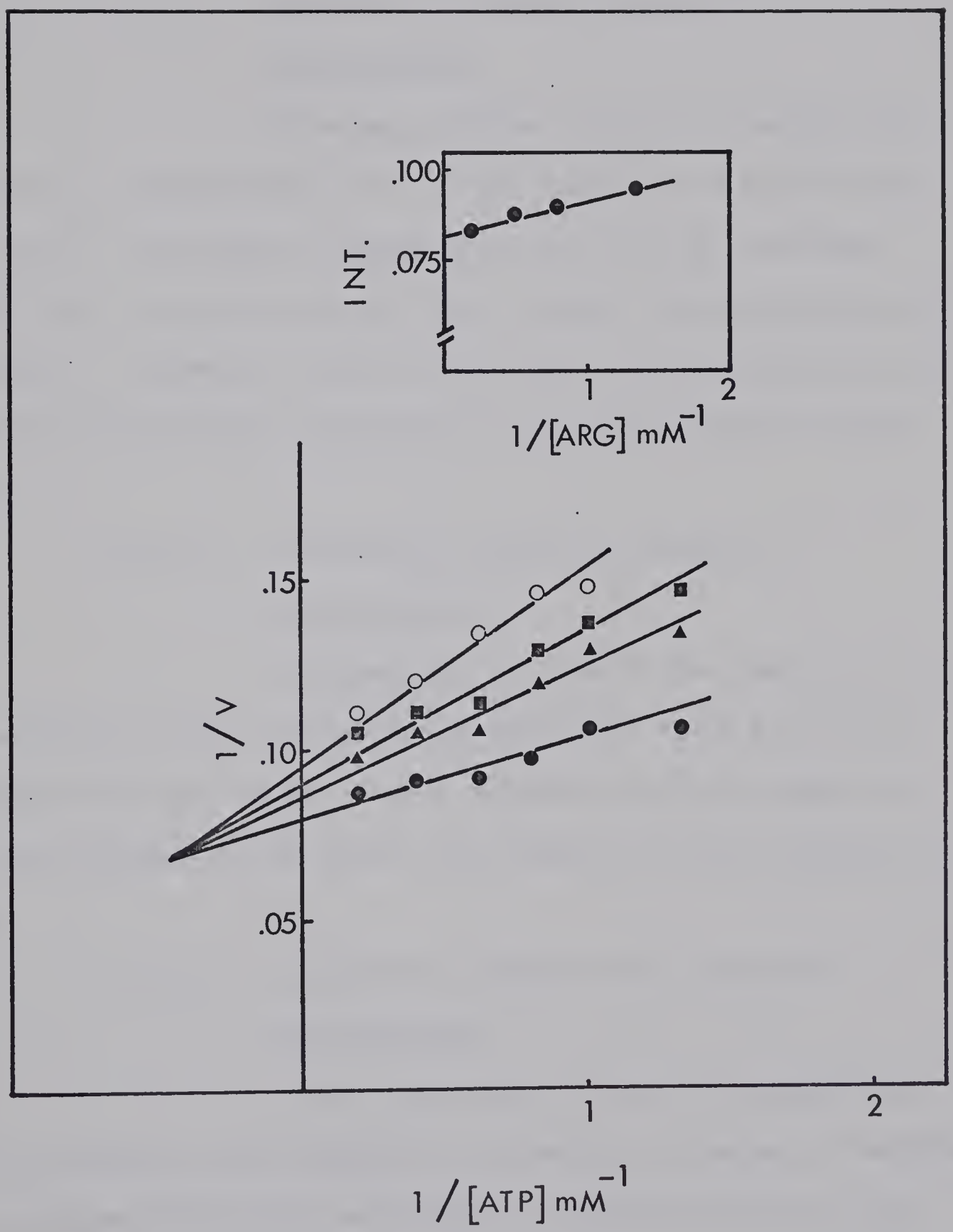
-●- 5.0 mM

-▲- 2.0 mM

-■- 1.25 mM

-o- 0.75 mM

v = µmoles AP/min/mg protein.


.100
.075
INT.
1
2
1/[ARG] mM^{-1}
.15
.10
.05
1/V
1
2
1/[ATP] mM^{-1}

experiment was done at pH 8.3.

4.12. Product inhibition studies

4.12.1. ATP synthesis

4.12.1.1. Inhibition by ATP with varied AP concentrations

The assay solution contained 0.9 mM ADP, 9.0 mM Mg^{++}, varied concentrations of AP, 0 mM, 7.2 mM, or 15 mM ATP in 50 mM Tris, pH 7.2. The amount of enzyme used was 0.2 µg in a reaction volume of 0.5 ml. Reaction time was 5 min. Double reciprocal plots are shown in Fig. 17. Addition of ATP to the reaction mixture affected both the slope and the intercept of each line. The lines intersect left of the Y-axis.

4.12.1.2. Inhibition by ATP with varied ADP concentrations

The experiment was done in the same way as in 4.12.1.1 except that the concentration of ATP was 0, 4, or 8 mM. The concentration of AP was 2.5 mM. Double reciprocal plots are shown in Fig. 18. The presence of ATP affects the slope but not the intercept of each line.

4.12.1.3. Inhibition by arginine with varied AP concentrations

In this experiment, it was not possible to measure the velocity of ATP synthesis by estimating the amount of arginine produced, because of the high concentration of arginine present in the reaction mixture relative to the arginine produced during the reaction. An alternate assay method as described in section 3.2.1.1 which measured the ATP produced was used.

Fig. 17. Inhibition of ATP synthesis by ATP with AP as the varied substrate. The concentration of ADP was 0.9 mM, Mg^{++} was 9.0 mM. ATP concentrations were:

-●- 0.0 mM

-▲- 7.2 mM

-■- 15.0 mM

v = µmoles ATP/min/mg protein.

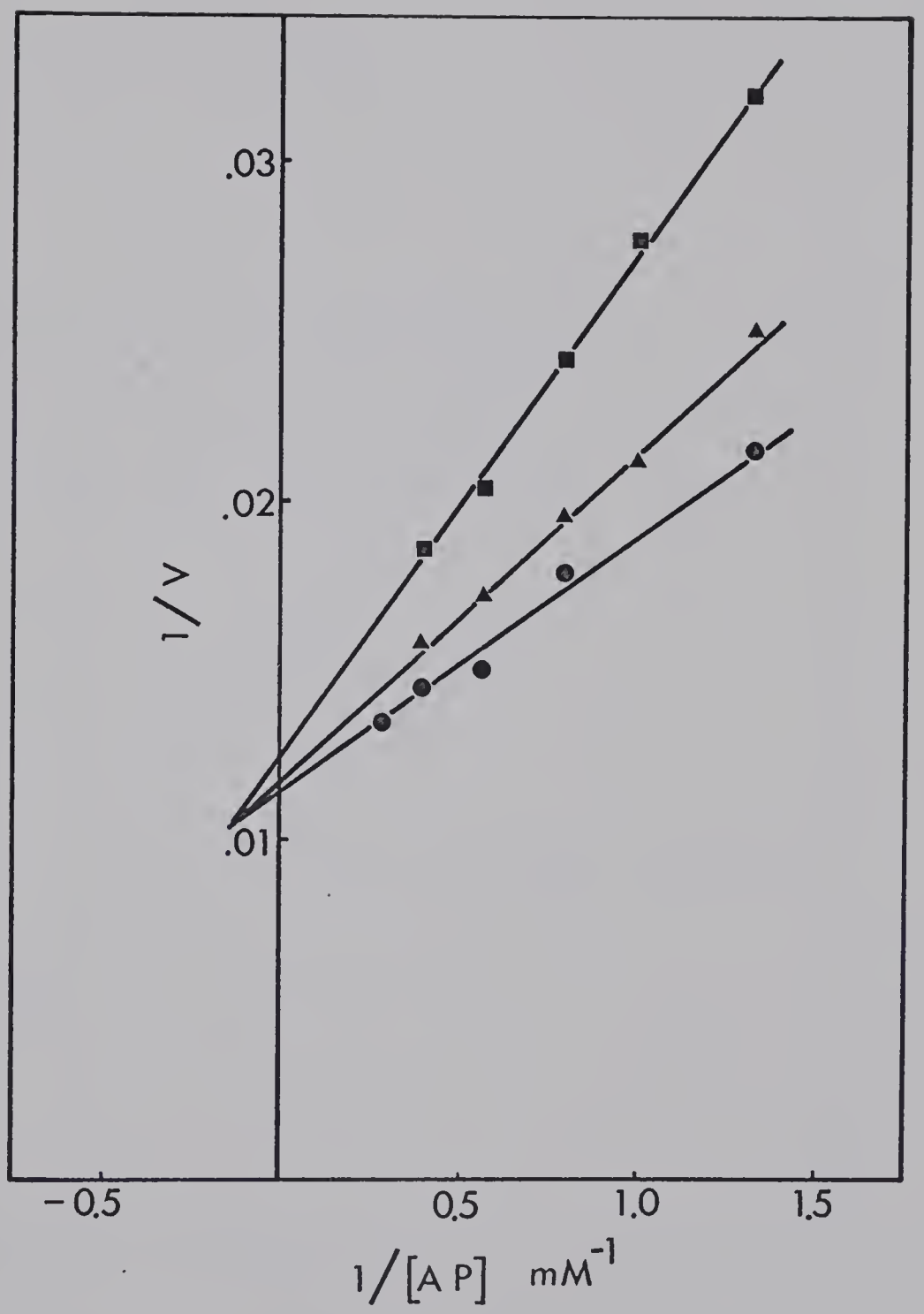

.03
.02
.01
1/V
-0.5
0.5
1.0
1.5
1/[A P] mM^{-1}

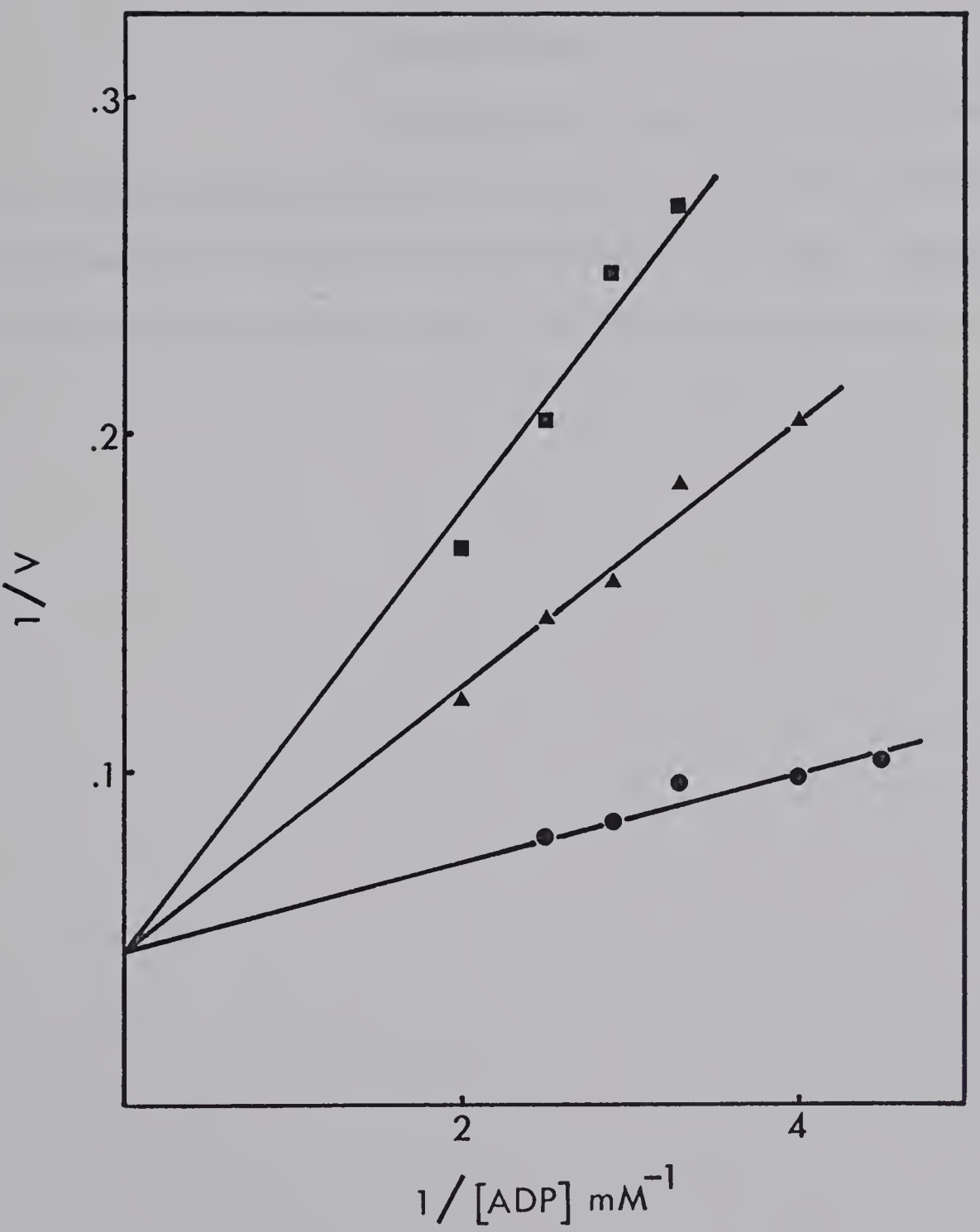
Fig. 18. Inhibition of ATP synthesis by ATP with ADP as the varied substrate. Concentration of Mg^{++} was ten times that of ADP, arginine was 2.5 mM. The concentrations of ATP were:

-●- 0.0 mM

-▲- 4.0 mM

-■- 8.0 mM

v = µmoles ATP/min/mg protein.


.3
.2
.1
1/v
2
4
1/[ADP] mM^{-1}

The assay solution contained 0.9 mM ADP, 9.0 mM Mg^{++}, and varied concentrations of AP. The concentrations of ATP were 0 mM, 7.2 mM, and 15 mM. Reaction time was 10 min and APK used was 0.2 µg. Double reciprocal plots of the data are shown in Fig. 19.

4.12.2. AP synthesis

4.12.2.1. Inhibition by ADP with varied arginine concentrations

The assay solution contained 5.0 mM ATP, 10.0 mM Mg^{++}, and varied concentrations of arginine in 50 mM Tris buffer, pH 7.2. The concentrations of ADP were 0 mM, 0.5 mM and 1.0 mM. Double reciprocal plots of the data are shown in Fig. 20. The lines intersect left of the Y-axis.

Fig. 19. Inhibition of ATP synthesis by arginine with AP as the varied substrate. The concentration of ADP was 1 mM, Mg^{++} was 10 mM. Arginine concentrations were:

-●- 0.0 mM

-▲- 1.25 mM

-■- 2.5 mM

v = µmoles ATP/min/mg protein.

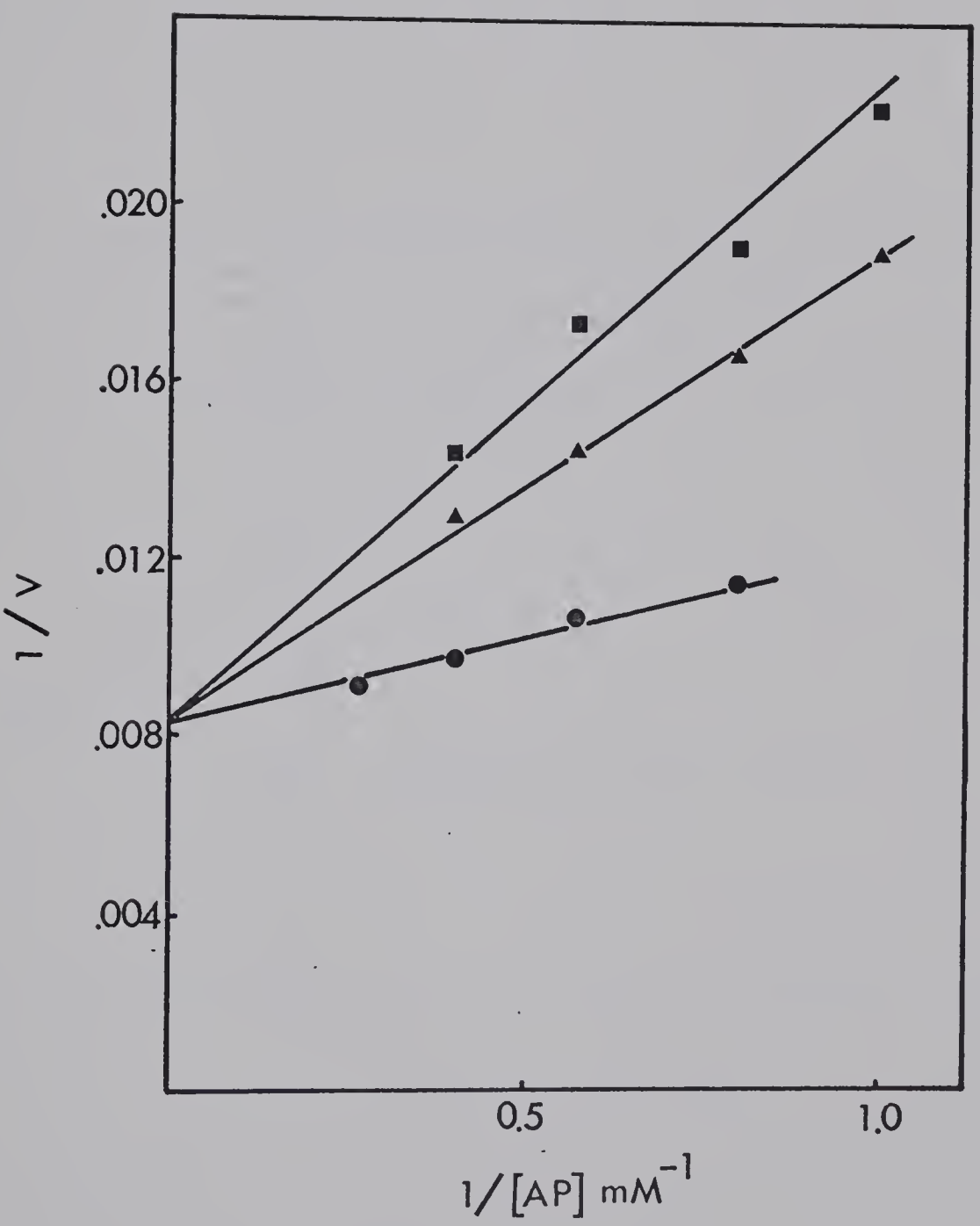

.020
.016
.012
.008
.004
1 / v
0.5
1.0
1/[AP] mM^{-1}

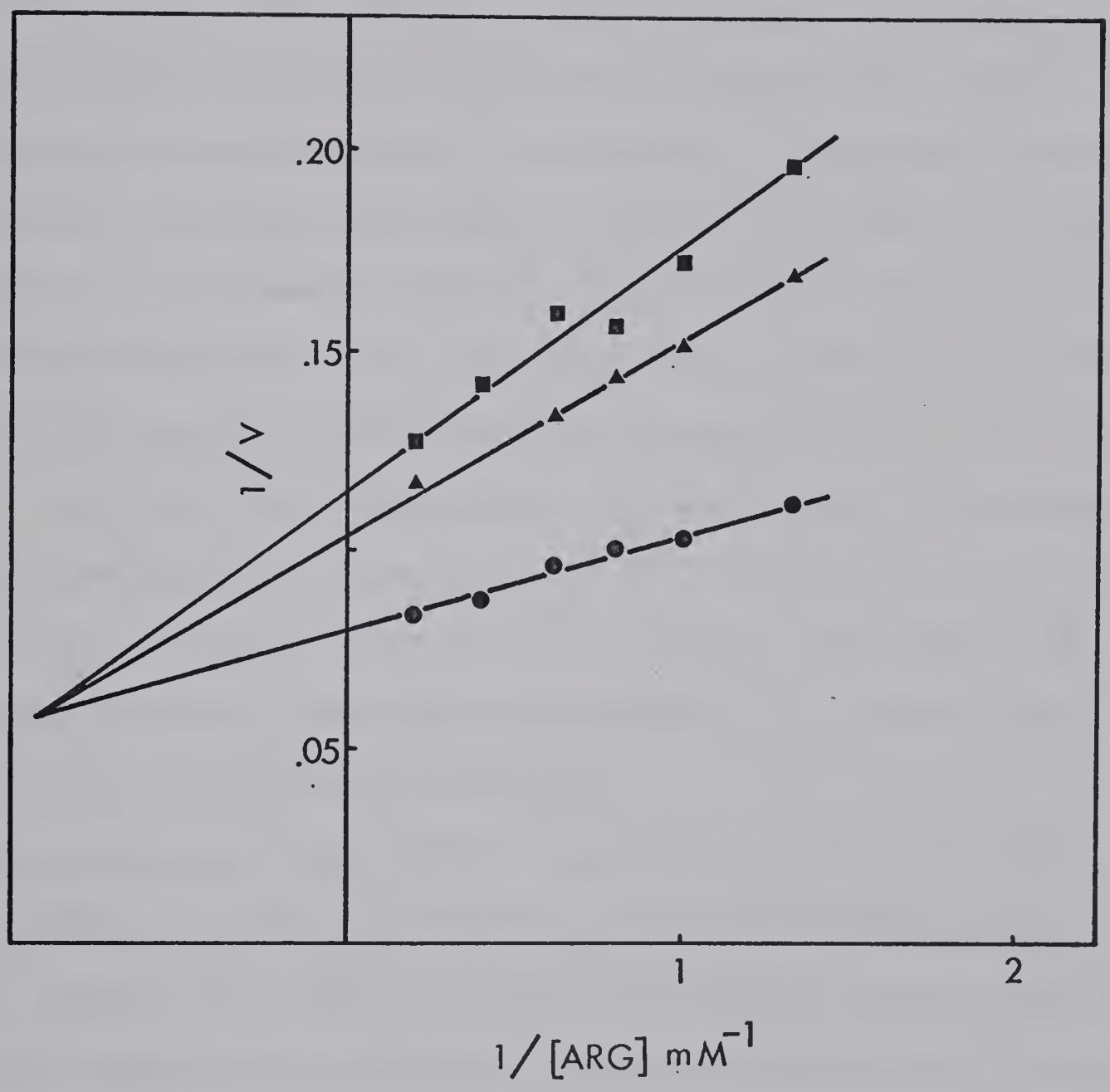
Fig. 20. Inhibition of AP synthesis by ADP with arginine as the varied substrate. The concentration of ATP was 5.0 mM, Mg^{++} was 10.0 mM. The concentrations of ADP were:

-●- 0.0 mM

-▲- 0.5 mM

-■- 1.0 mM

v = μmoles AP/min/mg protein.


.20
.15
.05
1/V
1
2
1/[ARG] mM^{-1}

5. DISCUSSION

AP can be obtained by chemical synthesis (Thiem *et al.*, 1962; Cramer *et al.*, 1962; and Marcus and Morrison, 1964), enzymatic synthesis (Marcus and Morrison, 1964), or by extraction from some suitable source such as crustaceans (Ennor *et al.*, 1956). Chemical synthesis either gives poor yield (Thiem *et al.*, 1962; Marcus and Morrison, 1964) or involves the laborious synthesis of intermediate compounds (Cramer *et al.*, 1962). My attempt to isolate AP from 200 g of tail muscles of commercially available live lobsters according to the method of Ennor *et al.* (1956) was rather unsuccessful, possibly due to the poor condition of the lobsters used. Using APK purified from honeybee thoraces, AP was synthesized from ATP and arginine following the method of Marcus and Morrison (1964). The AP obtained was then converted to BaAP for storage. Descending paper chromatography in a propanol-ammonia-water solvent revealed that the hydrolyzed BaAP contained arginine (Fig. 1) and the assay for Pi was positive. The ratio of Ba, bound-arginine, bound-Pi and water was 0.83:0.92:1:1. Traces of free arginine and Pi were detected in the BaAP.

Using sedimentation and diffusion experiments, Elodi and Szorényi (1956) estimated the molecular weight of APK from *Potamobius astacus* to be 43,000. Virden *et al.* (1966) concluded from estimates obtained with ultracentrifugal analysis, gel filtration, and density-gradient centrifugation, that the molecular weight of the enzyme from *Homarus vulgaris* was 37,000. Blethan and Kaplan (1968) estimated the molecular weights of APK from several arthropods by gel chromatography and obtained estimates from 35,000 ± 2,000 to 38,000 ± 2,000. Moreland and Watts (1967) discovered the existence of two forms of APK in some molluscs; one with a molecular weight

of 40,000 and the other 80,000. From the distribution of the isoenzymes in different muscle tissues, they suggested that different forms of enzyme were associated with different muscle functions and structures. Regnouf *et al.* (1969) showed that APK from *Homarus vulgaris* had a molecular weight of 43,000 and consisted of a single polypeptide chain, whereas APK from the annelid *Sipunculus nudus* with molecular weight of 86,000 was a dimer. Oriol *et al.* (1970) again showed that APK's from lobster and crab with molecular weights of about 40,000 were monomers. Robin *et al.* (1969) identified an APK from the polychaetes *Sabella pavonia* and *Spirographis spallanzanii* with a molecular weight of 160,000. Thus various workers have shown that there are at least three forms of APK in invertebrates, a monomer with a molecular weight of about 40,000, a dimer, and a tetramer, with corresponding molecular weights. In the present study, gel chromatography indicated that only one form of APK was present in honeybee thoraces, and the molecular weight of the enzyme was estimated to be 36,000 ± 3,000.

From the inhibition of the lobster APK by iodoacetamide, Ruiz Cruz *et al.* (1963) indicated that the enzyme contained a reactive sulfhydryl group which was essential for activity. Based on the reaction of the lobster APK with p-chloromercuribenzoate and N-ethylmaleimide, Pradel *et al.* (1964) showed that there were 6 -SH groups per molecular weight of 43,000. A similar result was obtained by Virden and Watts (1966) who showed that there were 5 reactive -SH groups per molecular weight of 37,000. Observing that one of the -SH groups was essential for enzyme activity and was much more reactive than the others, they concluded that it probably formed part of the catalytic mechanism at the single catalytic site. No detailed experiments on -SH groups were done in the present study. However, the activation of the enzyme by sulfhydryl compounds (Fig. 6) indicates that

the integrity of one or more of the -SH groups is important to enzyme activity.

Like other kinases, APK requires either Mg^{++} or Mn^{++} for its activity. As with the *Homarus vulgaris* enzyme (Virden *et al.*, 1965), APK from honeybees was also activated by Ca^{++} and Co^{++}.

When the effect of Mg^{++} concentration was studied at three ATP concentrations, it was apparent that the optimum ratio of Mg^{++}:ATP was 1:1 (Fig. 12). Similar observations have been made of other APK (Griffiths *et al.*, 1957b), creatine kinase (Kuby *et al.*, 1954), fructokinase (Hers, 1952), hexokinase (Liebecq, 1953), gluconokinase (Leder, 1957), phosphohexokinase (Lardy and Parks, 1956) and actomyosin ATPase (Perry and Grey, 1956). It is generally believed that a Mg-ATP complex is formed which can bind to the catalytic site of the enzyme molecule.

Depending on the conditions and the method used, various workers have estimated the Mg-ATP complex to be 2 to 18 times more stable than the Mg-ADP complex (Smith and Alberty, 1956; Nanninga, 1957; Walaas, 1958; Burton, 1959; Noda *et al.*, 1960; O'Sullivan and Perrin, 1964). This difference in stability between the two complexes might be one of the reasons that the optimum ratio of Mg^{++}:ADP is higher than that of Mg^{++}:ATP.

The enzyme is quite specific with respect to the nucleotide substrate. ATP cannot be substituted for by UTP, CTP, GTP, or ADP. It is less specific with the guanidino substrate. The enzyme is able to phosphorylate L-arginine methyl ester and to a much lesser extent, D-arginine. APK from *Sabella pavonina* was reported to show significant activity with D-arginine (28% of the activity with L-arginine) and those from *Maia squinado*, *Eupagurus bernhardus*, *Pecten maximum*, *Polycelis cornuta*, *Myxicola infundibulum*, and *Holothuria forskali* were also reported to have some activity with D-arginine

(1-7% of the activity with L-arginine) (Virden and Watts, 1964).

The pH optimum for ATP synthesis was 7.2-7.3 and that for AP synthesis was 8.3-8.4. Morrison *et al.* (1957) reported a pH optimum of 6.6-7.0 and 8.4-8.5 for ATP and AP synthesis, respectively, with APK from *Jasus verreauxi*. Virden *et al.* (1965) reported a pH optimum near pH 8.1 for AP synthesis with APK from *Homarus vulgaris*. Similar pH activity curves were observed for creatine kinase by Kuby *et al.* (1954) in which the pH optimum was around 7.0 and 9.0 for ATP and CP synthesis, respectively. However, Watts *et al.* (1968) observed that activity of an APK from the protozoan *Stentor coeruleus* did not alter significantly between pH 6.5 and 9.5.

The initial velocities of the reactions in the direction of ATP synthesis and AP synthesis were determined by varying one substrate while the other substrate was held fixed. The double reciprocal plots of the data from each experiment gave a family of intersecting lines. Such results indicate that the reaction mechanism is a sequential one in which both substrates are added onto the enzyme before the products are released. The results from product inhibition studies rule out an ordered sequential mechanism since then only one pair of substrate-product would show competitive inhibition. The fact that the double reciprocal plots are linear at all concentrations of the fixed substrate indicates that the mechanism is of a rapid equilibrium type in which all steps are in equilibrium except the step or steps involved in transphorylation. The mechanism of the enzyme is illustrated schematically in Fig. 21. The initial velocity of such a mechanism can be described by the following equation:

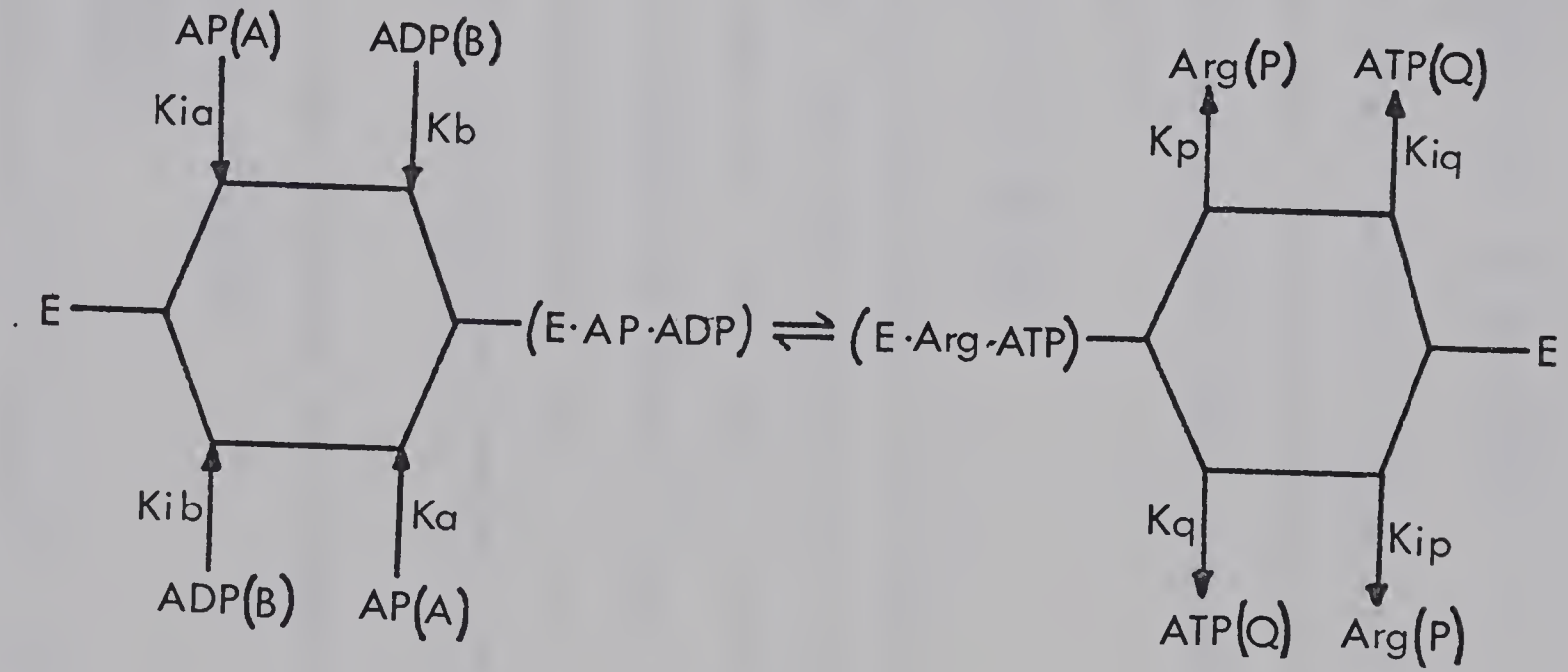
$$v = \frac{V_1 AB}{Kia\ Kb + KbA + KaB + AB} \qquad (1)$$

Fig. 21. Schematic representation of the mechanism of arginine kinase.

E - enzyme

Kix - dissociation constant of X from the EX complex

Kx - dissociation constant of X from the central complex


AP(A)
ADP(B)
Kia
Kb
E
(E·AP·ADP)
Kib
Ka
ADP(B)
AP(A)
Arg(P)
ATP(Q)
Kp
Kiq
(E·Arg·ATP)
E
Kq
Kip
ATP(Q)
Arg(P)

where A and B are the substrate concentrations, Kia is the inhibition constant of A, Ka and Kb are the Michaelis constants of A and B respectively, and V_1 is the maximum velocity of the forward reaction. The reciprocal form of equation (1) can be written as:

$$\frac{1}{v} = \frac{Ka}{V_1}\left(1 + \frac{Kia\ Kb}{KaB}\right)\left(\frac{1}{A}\right) + \frac{1}{V_1}\left(1 + \frac{Kb}{B}\right) \qquad (2)$$

A plot of 1/v against 1/A would yield a series of intersecting lines. The Vmax and the Michaelis constants can be conveniently obtained from a plot of Y-intercept against 1/B using data from the double reciprocal plots. The equation for the intercept replot is:

$$\text{Intercept} = \frac{1}{V_1} + \frac{Kb}{V_1}\left(\frac{1}{B}\right) \qquad (3)$$

Thus, the intercept at the vertical axis would give $1/V_1$, and that at the horizontal axis would give -1/Kb. The same argument can be applied when 1/B instead of 1/A is plotted against 1/v. The values for the apparent Km and apparent Vmax for ATP and AP synthesis are given in Table 4 and Table 5 respectively. The values are calculated from Figs. 13, 14, 15, and 16. The true Vmax, Michaelis constants and inhibition constants are given in Table 6.

For a rapid equilibrium, random mechanism, the Haldane relationship can be calculated from the following equation:

$$Keq = \frac{V_1\ Kip\ Kq}{V_2\ Kia\ Kb}$$

The value obtained for this reaction at pH 7.2 is about 80. This value is much higher than the reported value of 3.7 ± 0.04 of Smith and Morrison (1969) (calculated from the reciprocal of their data of 0.27 ± 0.04), who carried out their experiment at pH 8.0. The difference in pH's at which the experiments were run is probably the main reason for the discrepancy,

Table 4. Values for apparent Km and apparent Vmax in the direction of ATP synthesis, calculated from initial velocity data.

AP (mM)	App. Km for ADP (mM)	App. Vmax (μmoles ATP/min/mg protein)
5.0	0.21	114.0
3.5	0.204	96.2
2.0	0.204	66.7
1.0	0.196	61.7

ADP (mM)	App. Km for AP (mM)	App. Vmax (μmoles ATP/min/mg protein)
0.45	0.69	100.0
0.27	0.80	96.2
0.124	1.06	70.5
0.079	1.22	59.5

Table 5. Values for apparent Km and apparent Vmax in the direction of AP synthesis, calculated from initial velocity data.

ATP (mM)	App. Km for arginine (mM)	App. Vmax (μmoles AP/min/mg protein)
5.0	0.26	12.0
2.0	0.33	10.7
1.25	0.37	10.0
0.75	0.44	9.1

Arg. (mM)	App. Km for ATP (mM)	App. Vmax (μmoles AP/min/mg protein)
5.0	0.30	12.7
2.0	0.45	11.8
1.25	0.50	11.2
0.75	0.63	10.6

Table 6. Kinetic constants for APK catalyzed reactions.

ATP synthesis	
Maximum velocity (V_1)	137 μmoles ATP/min/mg protein
Michaelis constants:	
(a) AP (Ka)	1.3 mM
(b) ADP (Kb)	0.08 mM
Inhibition constants:	
(a) AP (Kia)	2.0 mM
(b) ADP (Kib)	0.23 mM
AP synthesis	
Maximum velocity (V_2)	12.2 μmoles AP/min/mg protein
Michaelis constants:	
(a) Arg (Kp)	0.19 mM
(b) ATP (Kq)	0.48 mM
Inhibition constants:	
(a) Arg (Kip)	1.0 mM
(b) ATP (Kiq)	2.38 mM

since this would reverse the favoritism of the reactions by pH. When the *in vivo* situation is considered, a high Haldane value in favor of ATP synthesis is advantageous to the insect, since the most critical function of this enzyme is to regenerate ATP as rapidly as it is used, before it could be supplied by glycolysis or oxidative phosphorylation.

The product inhibition studies revealed that arginine was competitive with AP but non-competitive with ADP, while ATP was competitive with ADP and non-competitive with AP. These relationships suggest that there are two binding sites at the catalytic center, one for the guanidine substrate, the other for the nucleotide substrate.

The pH optima of the ATP synthesis and AP synthesis reactions are different. Thus, the pH of the medium would play an important role in controlling the relative rates of the reactions. Lowering the pH from the slight alkaline range to neutrality would favor the synthesis of ATP and inhibit the synthesis of AP.

The Mg^{++} concentration may also be important in regulation of the reactions. Since Mg-ATP complex is much more stable than Mg-ADP complex, during resting metabolism when ATP level is relatively high most of the available Mg^{++} would be bound to ATP. However, as soon as muscular activity starts, Mg-ATP is utilized and ADP and Mg^{++} are formed. Besides upsetting the equilibrium, the breakdown of ATP would supply Mg^{++} to ADP to form Mg-ADP complex which would in turn be phosphorylated by APK with the phosphate from AP. However, since the optimum ratio of Mg^{++}:ADP for the reaction is 4:1, it would require more Mg^{++} ions than those released from the Mg-ATP complex. It is possible that some bound Mg^{++} is released at the onset of muscular activity so that optimal rate of ATP synthesis can be reached.

Despite the Haldane relationship of 80:1 in favor of ATP synthesis at pH 7.2, ATP and AP are present in more equal concentrations in insect thoracic muscles: Sacktor and Hurlbut (1966) reported that resting flight muscle of blowfly contained 3.1 µmoles AP and 6.9 µmoles ATP per g, wet weight, and Di Jeso *et al.* (1967) reported that the thoracic muscles of housefly contained 0.55 µmoles ATP and 2.98 µmoles AP per g, wet weight. There are several possible ways to prevent the AP stored in the muscle from being converted to ATP when it is not needed. A low concentration of Mg^{++}, as has been discussed earlier, would probably inhibit synthesis of ATP, since most of the available Mg^{++} would be present as Mg-ATP ccmplex owing to its higher stability compared to Mg-ADP complex. Another possibility is that a certain fraction of the AP synthesized may be compartmentalized in the muscle away from the APK. Some evidence for the compartmentalization of AP has been found by Seraydarian and Kalvaitis (1964) in *Mytilus californianus* muscle. Using two-dimensional chromatography, they found three distinct fractions of AP which they designated as AP1, AP2, and AP3. AP1 was free while AP2 was evidently and AP3 apparently bound to Ca^{++}. The incorporation of P^{32} was first into AP1, followed by AP2 and AP3. Chromatographically distinct AP was also observed in, among other organisms, the cockroach *Periplaneta americana*. Ca^{++} has been found to activate phosphorylase *b* kinase and α-glycerophosphate dehydrogenase, both of which are considered to be among the regulatory enzymes in glycolysis (Sacktor, 1970). As soon as free AP is used in producing ATP during muscular activity, Ca^{++} bound AP is released and the Ca^{++} freed. This freed Ca^{++} may be one of the sources of the Ca^{++} required for the activation of the glycolytic enzymes.

From the ratio of the concentrations of secondary phosphagen to ATP in the muscle tissues, it appears that the role of APK in insects may not

be as important as its counterpart in vertebrates, namely, creatine kinase. However, its significance in muscular activity cannot be totally ignored. The supply of stored ATP in muscles is limited. In muscles such as flight muscles in Diptera and Hymenoptera, and hindleg muscles of grasshoppers, the rate of energy utilization during activity is high, and the ATP in the muscle would be used up rapidly. Glycolysis and oxidative phosphorylation involve a series of enzyme catalyzed reactions which take a definite amount of time. On the other hand, phosphorylation of ADP by AP requires only one step which can readily be achieved. It is therefore reasonable to believe that AP plays an important role in maintaining a constant supply of ATP during the initial phase of muscular activity. However, the significance of APK in insect flight metabolism has so far been neglected. Sacktor (1970) in his review on insect flight muscle did not discuss the role of APK in flight muscles. Virtually no detailed studies have been made on the enzyme from an insect source. Detailed studies on the enzyme from insects not only can supplement our present knowledge of the muscle metabolism of the largest class of animals, they may also be of practical importance. It is possible that APK may serve as a suitable target for a selectively toxic material since the enzyme is not found in vertebrates.

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